Some systematic implications of the antigenicity of external mucus from British lampreys.

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SOME SYSTEMATIC IMPLICATIONS OF THE ANTIGENICITY OF EXTERNAL MUCUS FROM BRITISH LAMPREYS

submitted by Elizabeth Anne Pass

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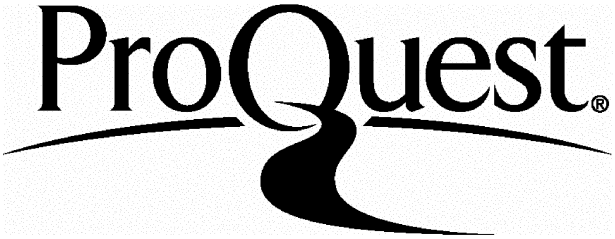

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TABLE OF CONTENTS

		Page
SUMMARY		iv
I.	INTRODUCTION	1
A.	Phylogeny of lampreys	1
B.	Distribution of lampreys	9
C.	General Biology	12
1.	Appearance	12
a)	adult lampreys	12
b)	the ammocoete	13
2.	Life Histories	13
D.	British lampreys	18
1.	Petromyzon marinus	18
2.	Lampetra fluviatilis	19
3.	Lampetra planeri	20
E.	Methods of identification of ammocoetes	22
1.	'Biological differentiation'	22
2.	External morphology	23
3.	Internal morphology	23
F.	The immune response of lampreys	26
II.	MATERIALS AND METHODS	28
A.	Introduction	28
1.	The advantages of immunology in taxonomy	28
2.	Selection of antigen	30
B.	Collection of lampreys and field work	37
1.	Ammocoetes	37
2.	Adults	38
3.	Filming of spawning behaviour	40
4.	Redd measurements	41

		Page
C.	Collection of antigens	44
D.	The preparation of antisera	48
E.	Immunodiffusion	51
	1. The 'Ring' test	51
	2. Diffusion in gels	51
	i) Double gel diffusion techniques	54
	ii) Immunoelectrophoresis	54
F.	Immunofluorescence techniques	57
G.	Isolation of specific antibodies	60
H.	Thin layer chromatography	64

III. RESULTS.

A.	Observations on the spawning of lampreys	66
	1. The spawning of L. planeri	67
	2. The spawning of P. marinus	70
	3. The spawning of L. fluviatilis	73
	4. Artificial fertilisation of lamprey eggs	75
B.	Redd measurements	79
C.	Gel diffusion	83
D.	Immunoelectrophoresis	85
	1. Variability of patterns	85
	a) Sera	85
	b) Antigens	88
	c) Technique	88
	2. Composite pattern	88
	a) adult and ammocoete comparisons	89
	i) fluviatilis	89
	ii) planeri	90
	iii) marinus	90

Page

b) Sex differences 91

3. Specific patterns 91

a) marinus 91

b) fluviatilis/planeri 94

c) Teleosts 95

4. Summary of immunoelectrophoretic results 95

E. Antibody preparation and fluorescent labelling 97

F. Thin layer chromatography results 98

IV. DISCUSSION 99

A. Identification 99

B. Lampreys and speciation 107

1. 'Paired species' 107

2. Species definitions and concepts 110

3. The reasons for speciation 115

4. Methods of speciation 119

a) the origin of diversity 119

b) isolating mechanisms 125

c) geographical isolation 137

d) rates of speciation 148

5. Lampreys and orthodox speciation theories 153

6. Models of speciation of lampreys 165

Scheme I. transition through praecox 172

Scheme II. direct transition from fluviatilis to planeri 174

V. REFERENCES 185

SUMMARY

1. This thesis outlines the phylogeny, distribution, biology and life histories of lampreys, with particular reference to the identification of British lampreys.

2. Immunological techniques of identification were explored, using the external mucus and other antigens, and the results discussed.

3. The implications of the results for the taxonomy of the lampreys led to field observations of their spawning behaviour which is described.

4. The identification of Petromyzon marinus presents no difficulties, but the difference between Lampetra fluviatilis and L. planeri is so small as to cast doubts on their taxonomic status as species.

5. In view of these doubts, species concepts, isolating mechanisms and modes of speciation are discussed, particularly as they relate to possible mechanisms of lamprey speciation.

6. Models of the derivation of non-parasitic lampreys from their 'paired' parasitic forms are suggested and discussed.

7. Ways of testing the models by hybridisation and marking experiments are proposed.

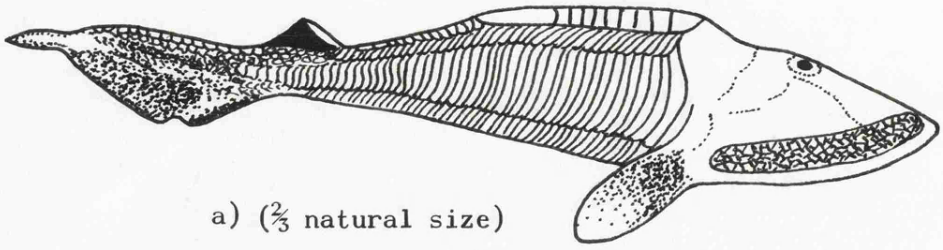
I. INTRODUCTION

The lamprey presents taxonomists with difficulties which have not been found in quite the same form in any other group of animals. This work is an attempt to resolve some of these problems and to suggest possible explanations of the others. Immature lampreys are difficult to identify and an immunological technique has been explored to achieve their identification. The recognition of the separate taxonomic status of an animal implies that it has evolved with distinction from its contemporaries. Since this evolution has apparently occurred, postulates to explain the selective mechanism must be offered. Since such selection operates throughout the life of an animal which dies immediately after spawning, there must be consideration of its entire biology, hence some of the practical work has been on relevant aspects of its reproductive behaviour.

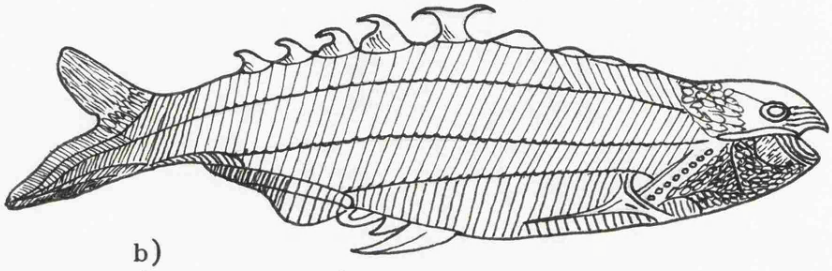
A. Phylogeny

Although it has been agreed that the Cyclostomes belong to the Class Agnatha, the relationship between the Petromyzonida and the Myxinoidea has been the subject of much discussion, as has the relationship between these two groups and the fossil Agnatha.

The term Agnatha has formerly been used to describe those true vertebrates that do not have jaws formed from the first two branchial arches, as are the jaws found in the Gnathostomes. The use of the word 'Agnatha' has been questioned by Jarvik (1964, 1968) and by Stensiö (1968) who contend that some of the fossil forms had a biting apparatus and all should therefore be referred to as the Cyclostomata, a term formerly reserved for the extant Agnathans, the Petromyzonida and the Myxinoidea. It is convenient however,



a) (⅔ natural size)



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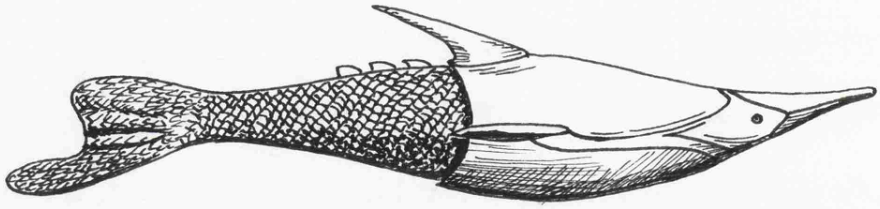


c) (1½ natural size)

Figure 1. a) Hemicyclaspis, a late Silurian Cephalaspid.
b) Birkenia, a Silurian Anaspid.
c) Pteraspis, a Devonian Pteraspid.

(Facing p. 2)

to retain a separate term for the living forms, so the word 'Agnatha' is here used to include all the conventionally jawless vertebrates - the Cephalaspidomorphi, the Pteraspidomorphi and the two living groups; which last two are herein referred to as the Cyclostomata.

The Agnatha are well represented in the fossil record, first appearing in the Ordovician as rather fragmentary fossils in freshwater facies. In the Silurian and Devonian a large number of forms occurred but disappeared during the Upper Devonian, presumably ousted by the increasingly diversified jawed fishes.

A classification of the Agnatha from Stensiö (1968) with taxonomic grades from Romer (1945) is given below.

Class.		AGNATHA
Sub-class	I.	Cephalaspidomorphi
Order		Osteostraci (= 'Cephalaspids')
Order		Anaspida
Order		Petromyzontida.
Sub-class	II.	Pteraspidomorphi
Order		Heterostraci
Order		Myxinoidea

The Cephalaspidomorphi are distinguished by the dorsal position of the single naso-hypophysial opening; the Pteraspidomorphi have single or double naso-hypophysial openings which are ventral or terminal on the snout and they may not have a pineal opening.

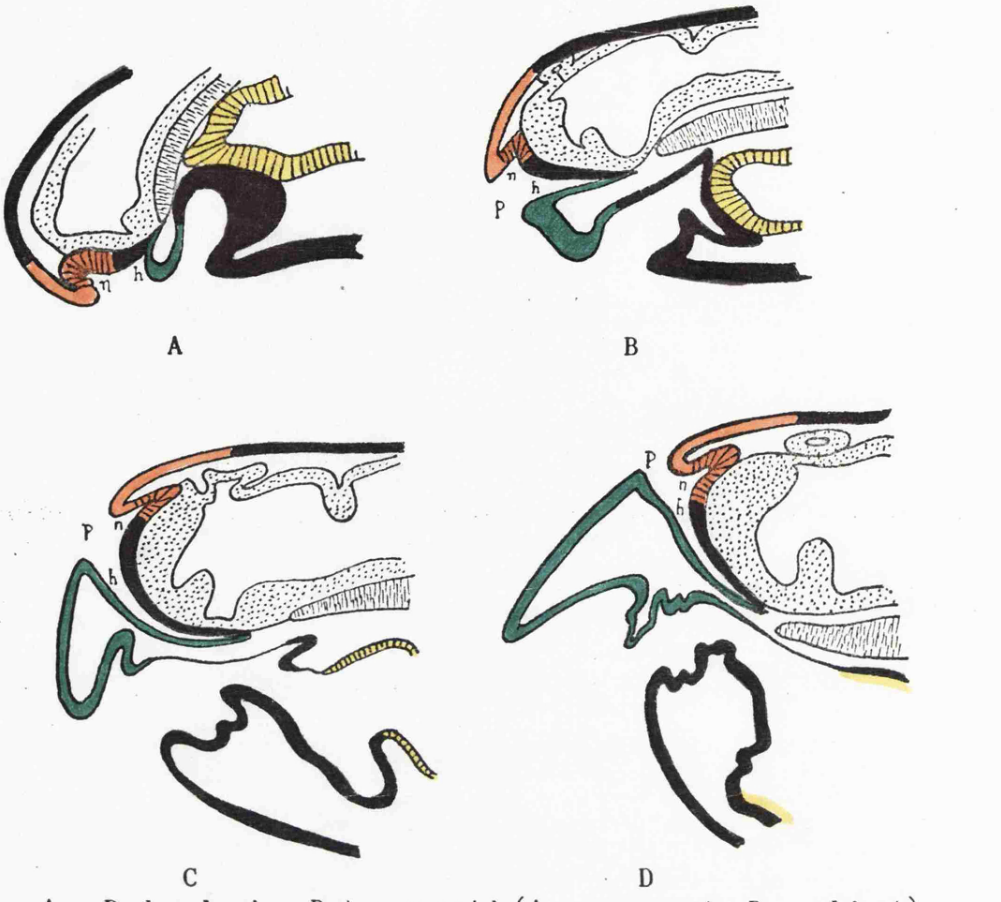
The Osteostraci (Fig. 1a) are found in the Silurian and Devonian. They were completely armoured with bone and a single massive head shield covered the dorsal head and 'shoulders' region. Paired fins were present in many forms but these are not homologous with the paired fins of the gnathostomatous fishes.

Most osteostracans showed considerable dorso-ventral flattening which, with the dorsal position of the eyes, indicates that they were bottom-dwellers. Romer (1945) suggested that the expanded branchial chambers indicated a filter-feeding habit, but Denison (1961) believed that most were too large and too active for this mode of feeding and that most were mud eaters.

The early Anaspids (Fig. 1b) also had a complete bony armour, but the head shield, where present, was formed by fused scale-like plates. Many later forms had lost most or all of their armour. Unlike the Osteostraci there were no paired fins and the body was not depressed. They were probably more active than the osteostracans but Denison (1961) suggested that they also were mud swallowers.

The earliest fragments of vertebrate fossil, from the Ordovician, are probably Heterostracan and although these forms (Fig. 1a) appear superficially similar to the Cephalaspidomorphi, they are probably not closely related. The armour was not of true bone and the paired naso-hypophysial openings were situated below an elongated snout, the rostrum. They were probably active swimming forms, being little flattened. Stensiö (1968) has interpreted the oral plates around the mouth as a mechanism capable of biting or nibbling but Denison (1961) suggested that these mouth parts were only capable of holding or grasping and that the Heterostraci were probably selective detritus feeders (compared with the suctorial feeding of the Cephalaspidomorphi).

The modern Cyclostomes and the Cephalaspidomorphi have long been regarded as monorhine from their single naso-hypophysial opening while the Heterostraci are classified as diplorhine. This division of the Agnatha has made the elucidation of the relationships between the fossil and extant Agnatha rather difficult.



KEY

p = naso-hypophysial opening.
n = true nasal opening.
h = hypophysial opening.
= unspecified ectoderm.
= brain.
= olfactory organ.
= cranial snout.
= visceral snout (= post-hypophysial fold).
= endoderm.
= notochord.

Figure 2. Median longitudinal sections of the snout in the named animals to show regional and developmental relationships. (All from Stensiö, 1968; A - D after Goodrich, 1909; E after Kupffer, 1900 & 1906.)

(Facing p. 4)

However, Stensiö (1968) has shown that the nasal capsule was originally paired in all Agnatha and that the single external opening in the Cephalaspidomorphi and the Cyclostomes is a result of the evolution of the anterior head region in these forms.

The 'snout' of the Agnatha is formed from two embryonic components, a superior cranial component and a ventral visceral part. Differential development of these components has produced the snouts found in adult fossil and extant Agnatha. Strahan (1958) has used the method of co-ordinate deformation of D'Arcy Thompson (1942) to produce predictions of the ontogenic development of the snout and Stensiö (1968) has used the same data to show the relationships among the Agnathan orders.

The original, most primitive condition (Fig.2a) is that found in the pro-ammocoete larvae of the lampreys at the stage when the olfactory organs first differentiate. At this stage the nasal openings (n) are separate from that of the hypophysis (h) and are ventrally situated just in front of the stomodaeum.

During the development of the Cephalaspidomorphi and the Petromyzonidae the fold of tissue between the hypophysial opening and the stomodaeum (the post-hypophysial fold) greatly enlarges (Fig. 2b & c), pushing the openings forward and then upward. Eventually in the adult (Fig. 2d) the openings lie dorsally with a single common exit, the naso-hypophysial opening.

The space into which the olfactory organs and hypophysis open, Stensiö's pre-nasal sinus, is in fact an extracephalic space, created solely by the post-hypophysial fold. The true nasal openings are originally paired and open into this space.

The forward evolution of the post-hypophysial fold is found in the Osteostraci, the Anaspida and the Petromyzonidae. In the

Myxinoidea both cranial and visceral components enlarge to the same forward extent or the visceral part is the shorter (Fig. 2e). The naso-hypophysial opening is formed in the same way as in Petromyzon, but opens terminally or ventrally. The Heterostracan snout shows the same development as that of the Myxinoids, but with the later development of the rostral cartilage in some forms (Fig. 2f).

Stensiö (1968) listed other characters that the Myxinoidea and the Heterostraci had in common and considered that the two groups were more closely related to each other than to any other Agnathan group, as did Strahan (1958) and Jarvik (1968).

The majority of Cephalaspids and Anaspids represented as Silurian or Devonian fossils were too specialised to have given rise to the Petromyzonidae, which probably arose from a less specialised Cephalaspidomorph type in the Ordovician or Cambrian. Such a type is perhaps represented by Jamoytius kerwoodi, White, a Silurian fossil showing a simple Cephalaspidomorph organisation. Jamoytius, classified by Ritchie (1968) as an Anaspid, possessed a simple cyclostome-like branchial basket and unlike the majority of Cephalaspidomorphi was not heavily armoured, although it did possess lateral trunk scales.

Ritchie considered the animal to be too long to filter-feed and the small mouth supports the view that it was a suctorial mud or detritus feeder.

The presence of lateral fin-folds and the occurrence of Jamoytius in the Silurian rather than earlier, probably precludes this species from being directly ancestral to the lampreys, but such ancestors were probably very similar and possibly closely related to Jamoytius.

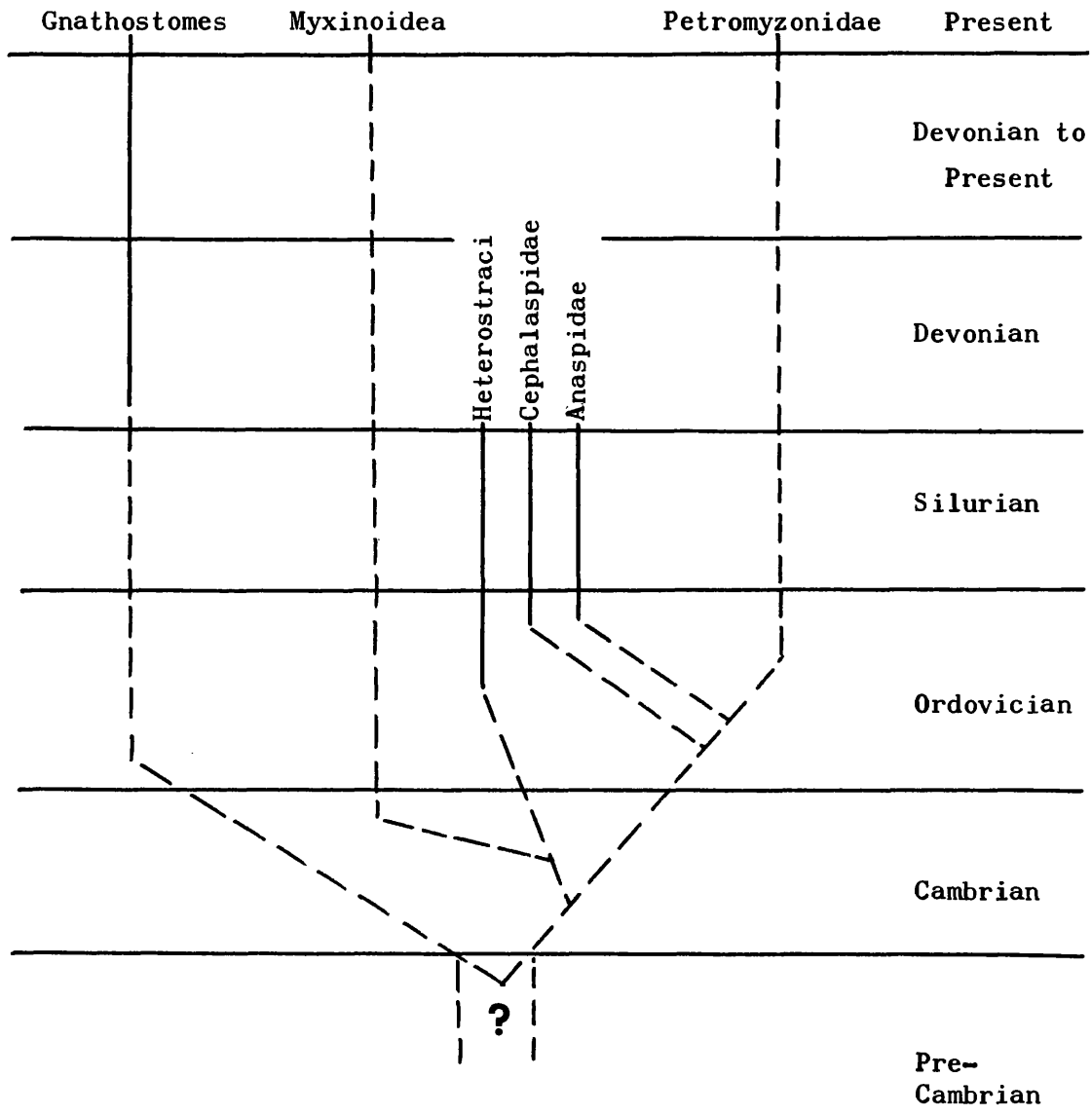
The only fossil lamprey so far found is Mayomyzon pieckoensis from the Carboniferous Pennsylvanian of the United States. Bardack and Zangerl (1968) have described this species which is in most ways very similar to the metamorphosing stages of modern lampreys, except that it is only one quarter to one half of the size of known modern macrophthalmia, i.e. the immediately post-metamorphic stage. Like modern macrophthalmia Mayomyzon possessed large, well developed eyes but in contrast there are no teeth. The presence of a piston was interpreted by Bardak and Zangerl as implying the possession of a rasping tongue, hence if this is so they probably had a parasitic mode of life.

Bardack and Zangerl have suggested that because of the many features that appear to have remained unchanged since the Carboniferous, the lampreys have a long pre-Carboniferous evolution, and that, because Mayomyzon shows no Myxinoid characters, the division between the Petromyzonidae and the Myxinoidea was already long established by this time. These conclusions agree well with those of Stensiö (1968) regarding the evolution of the Cyclostomes, but Bardack and Zangerl also suggest that the oral hood, which is the post-hypophysial fold, is a purely lamprey modification to the specific method of feeding; although Strahan (1958) and Stensiö (1968) have shown that the upper lip development has probably taken place in all Cephalaspidomorphi.

The lampreys are almost certainly an ancient and conservative group on this evidence. Jarvik (1968) believes that the Agnatha are in no way primitive compared with the Gnathostomata and that, in fact, many Gnathostomes show primitive features when compared with the Agnatha. He proposed that the Agnatha and the Gnathostomata are 'sister groups' (Hennig, 1966) and as such arose from

a common ancestor, probably in the Pre-Cambrian.

If this view is accepted, the evolution of the Agnatha can be represented by the diagram below. The absence of the Gnathostomata in the fossil record before the Devonian is probably due to the lack of massive bony structures.

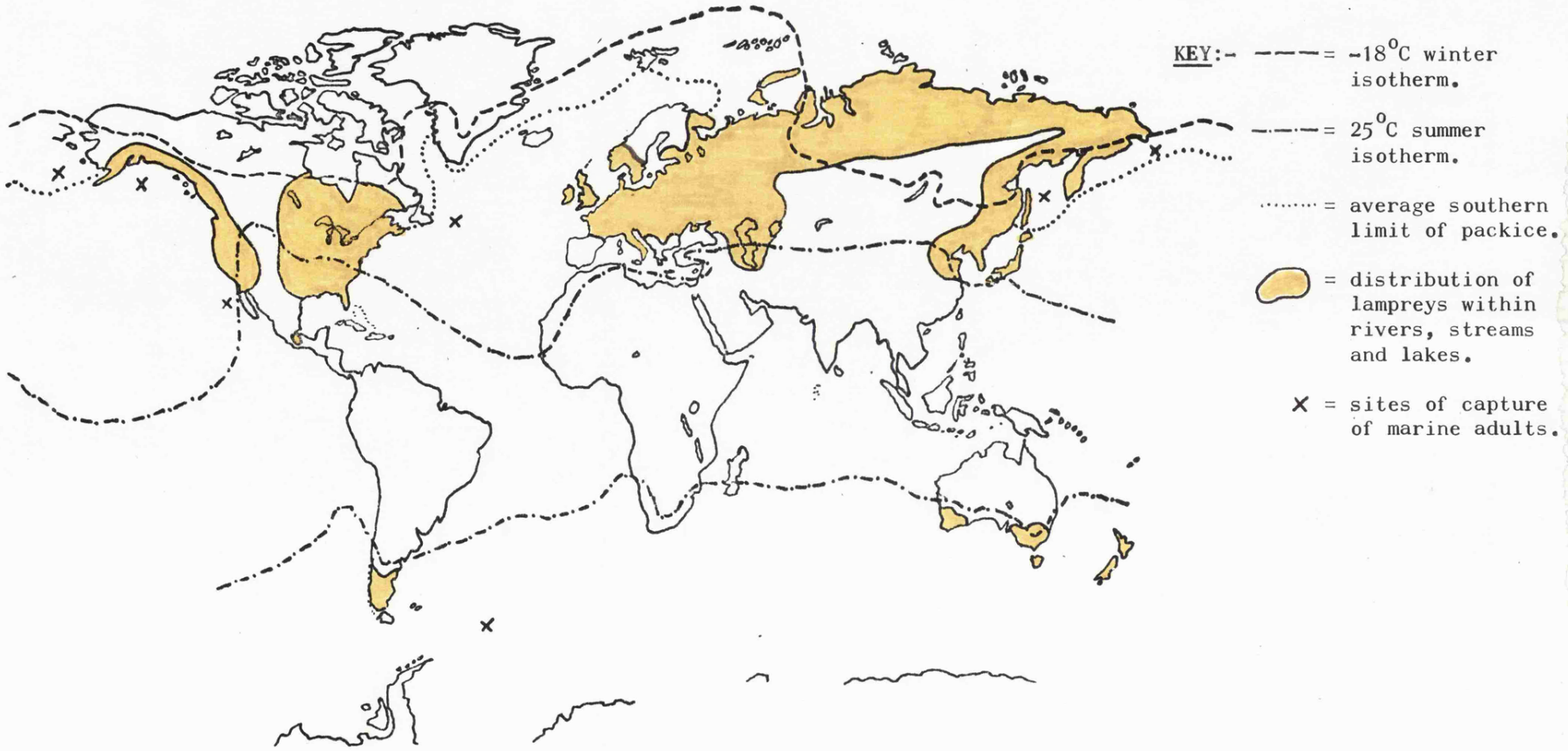


To sum up, the Petromyzonidae are derived from a simple, probably Anaspid-like stock at some time during the early Palaeozoic. Denison (1961) suggested that all of the Cephalaspidomorphi were suctorial feeders, probably eating mud. Ritchie (1968) has interpreted Jamoytius as a suctorial feeder on Dictyocaris, thin sheets of which are found pierced with round holes on the same horizon as

Jamoytius. Dictyocaris has been interpreted variously from an alga to a primitive crustacean, so its true nature is uncertain (Ritchie, 1968). It seems probable however, that the lampreys, arising from a Jamoytius-like ancestor, would have started as suctorial feeders, perhaps progressing to scraping algae or crustaceans from rock surfaces with a rasping tongue and thence to parasitism. If Jarvik's interpretation (1968) of the phylogeny of Gnathostomes and Agnathans is correct, it is possible that there were primitive unarmoured Gnathostomes at that time which would have formed suitable prey for an incipient parasite or predator.

The armour of Jamoytius was incomplete and many later Anaspids were unarmoured. The lampreys may have arisen therefore from a later unarmoured stock, or, more probably, from a partially armoured early line, losing their remaining armour on becoming actively swimming predators.

Figure 3. A map of the world showing the distribution of lampreys.



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B. Distribution

Extant lampreys are holarctic in distribution, occurring in rivers in most temperate regions. Figure 3 is a sketch map of lamprey distributions but, as many reports deal with a single site or a single river, it has been necessary to suggest a general distribution from these limited data.

The majority of species occur between the -18°C mid-Winter isotherm and the 25°C mid-Summer isotherm. This distribution may be a reflection of temperature tolerances or it may be that latitudes outside these limits do not have rivers with constant freshwater.

There are three types of life-histories among lampreys and apparent anomalies in distribution are perhaps better understood with reference to these life-histories. Lampreys have a larval life of up to six years in rivers or streams. The anadromous lampreys migrate to the sea after metamorphosis, returning to the rivers to breed after a feeding period of perhaps several years. The freshwater parasitic forms also migrate after metamorphosis but stay within freshwater, returning upstream to breed. Non-parasitic forms also migrate upstream to breed a few months after metamorphosis with no intervening adult feeding phase.

The lampreys of the Gulf States of the U.S.A. occur south of the Summer 25°C isotherm but all are freshwater parasitic or non-parasitic, and live in a region of high Summer rainfall (50mm average in July). Tetrapleurodon occurs well south of the Summer isotherm in Central Mexico but both species are freshwater only and are found in lakes and rivers at altitudes above 2000m, where average July temperatures are below 20°C.

With the exception of Tetrapleurodon, the most southerly species in the northern hemisphere are non-parasitic and they do not have

a parallel 'parental' parasitic species. For this reason and because of other 'degenerate' features (e.g. the loss of teeth in Okkelbergia) they are often regarded as 'relicts' from a period, probably during the Ice Age, when lampreys had a more tropical distribution. Okkelbergia occurs in the Mississippi basin, a region noted for its relicts of ancient gnathostome fish forms, for example, Polydon and Amia.

The distribution of the north Russian lampreys is puzzling. The anadromous form, Lampetra japonica septentrionalis, occurs up to the -18°C January isotherm, and not far beyond the southern limit of the pack ice. Presumably the pack ice, even after a cold Winter, has broken up by the time of the breeding migration. L. japonica kessleri, however, is recorded in rivers from the Ob to the Anadyr, in a region where the average January temperatures are -35°C (Lindberg and Legeza, 1959). In as a freshwater parasitic form it is hard to understand how this distribution of a form that would seem to be dependent upon flowing water for much of its life history is compatible with the average temperatures. However, most reports are merely records of its occurrence within a river system and all of the rivers of this coast rise far to the South where January temperature averages are up to -18°C. It is possible that adults migrate upriver for the Winter and if the majority of adults spawn up-river also, most ammocoetes would occur within this region of milder winters.

Little is known of the marine distribution of the anadromous lampreys. Zanandrea (1962) stated that Lampetra fluviatilis is found in inshore waters within the continental shelf areas while the larger Petromyzon marinus is more oceanic. This distribution is probably applicable to all anadromous lampreys with only the

TABLE 1. A summary of lamprey distribution continued.

Lamprey	Type	Distribution	References
Lampetra			
japonica sub-spp?	-	Novaya Zemlaya. River and Lake Naknek in south west Alaska.	Tambs-Lyche, 1963; Heard, 1966.
Geotria			
australis	a	Southern coasts of mainland Australia; Tasmania; New Zealand; Chile; Argentina and Falkland Islands. Marine phase caught off South Georgia Island.	Tickell, 1964; Permitin, 1966; Ivanova-Berg, 1968; Potter, 1968 Hubbs & Potter, 1971.
Mordacia			
mordax	a	South Eastern Australia and Tasmania.	Potter, 1968 Potter, Lanzing & Strahan, 1968; Hubbs & Potter, 1971.
praecox	n-p	New South Wales.	as mordax.
lapicida	a	Chile.	Hubbs & Potter, 1971.

This Table has been compiled from those references that gave the broadest information and it is not intended to be a detailed survey of lamprey distribution.

TABLE I. A summary of lamprey distribution continued.

Lamprey	Type	Distribution	References
Lampetra			
wilderi (= *lamotteni* = *lamottei*)	n-p	Southern New England from northern New York to Maryland; west to Wisconsin and Iowa.	Creaser & Hubbs, 1922; Hubbs & Potter, 1971; Vladykov, 1950.
reissneri	n-p	Rivers of northern west Pacific drainage, from river Anadyr in the north to Korea in the south, and both coasts of Japan.	Berg, 1931; Lindberg & Legeza, 1959; Lebedev *et al*, 1969; Hubbs & Potter, 1971.
zanandreai	n-p	Rivers of the Po Plain in northern Italy.	Vladykov, 1955; Hubbs & Potter, 1971.
fluviatilis	a	Western European rivers of Baltic, Atlantic and Mediterranean drainage, from Stavanger and the river Neva in the north to the rivers Tiber and Po in the south; not Iberia. Landlocked form in Lakes Ladoga, Onega and Mjösen.	Berg, 1932 & 1935; Zanandrea, 1959 Tambs-Lyche, 1963; Lebedev *et al*, 1969; Hubbs & Potter, 1971.
planeri	n-p	As *L. fluviatilis*. Also one report* from the river Congo.	Tambs-Lyche, 1963; Lebedev *et al*, 1969; Hubbs & Potter, 1971. *Pellegrin, 1923.
ayresi	a	Western North America, from northern British Columbia, and possibly south east Alaska, to California.	Vladykov and Follet, 1958; Vladykov, 1965; Hubbs and Potter, 1971.
richardsoni	n-p	British Columbia to Oregon, possibly south east Alaska.	Vladykov, 1965; Hubbs & Potter, 1971.
japonica septentrionalis	a	White Sea, Barents Sea and Ob Bay; rivers Tobol, Vyg, Onega, Dvina and Petschora.	Berg, 1931, 1932 & 1948.
japonica kessleri	f-p	In rivers from the Ob to the Anadyr and lakes and rivers of Sakhalin Island.	Berg, 1931 & 1932; Lindberg & Legeza, 1959; Hubbs & Potter, 1971.
japonica japonica	a	South from the river Anadyr to Pusan, including Japan. Yukon river in Alaska.	Creaser & Hubbs, 1922; Berg, 1931; Lindberg and Legeza, 1959; Hubbs & Potter, 1971.

TABLE 1. A summary of lamprey distribution continued.

Lamprey	Type	Distribution	References
Eudontomyzon			
danfordi	f-p	Danube basin.	Berg, 1931 & 1932; Hubbs & Potter, 1971.
vladykovi	n-p	Danube basin.	Hubbs & Potter, 1971.
mariae	n-p	Rivers Don, Danube, Dnestr, Dnepr, Kuban, and rivers of western Transcaucasia. Probably river Vardar in Macedonia. Rivers Neman and Upper Vistula of the Baltic Basin.	Berg, 1931 & 1932; Lebedev et al, 1969; Hubbs and Potter, 1971.
morii	f-p	Yellow Sea basin, Manchuria, Korea and Japan.	Berg, 1931; Lindberg & Legeza, 1959; Hubbs & Potter, 1971.
Tetrapleurodon			
spadiceus	f-p	Rio Lerma, Rio Duera, Lake Chapala, in the provinces of Jalisco and Michacoacan, western Mexico.	Alvarez del Villar, 1964; Hubbs & Potter, 1971.
geminis	n-p	As spadiceus.	As spadiceus.
Okkelbergia			
aepyptera	n-p	The Ohio river basin in eastern North America.	Creaser & Hubbs, 1922; Hubbs & Potter, 1971.
Entosphenus			
tridentatus	a	Pacific drainage; from Unalaska Island to Oregon in North America; Bering Island and southern Sea of Okhotsk in Asia. Marine phase caught in Bering Sea, Gulf of Alaska and off Baja California.	Berg, 1931; Svetovidov, 1948; Lindberg and Legeza, 1959; Abakumov, 1964; Hubbs, 1967; Hubbs & Potter, 1971.
lethophaga	n-p	Tributaries of upper Sacramento river and Lake Klamath drainage (Oregon).	Hubbs & Potter, 1971.

TABLE I. A summary of lamprey distribution. a = anadromous; f-p = freshwater parasitic; n-p = non-parasitic.

Lamprey	Type	Distribution	References
Ichthyomyzon			
unicuspis	f-p	Hudson Bay drainage; Great Lakes drainage; St. Lawrence basin; north eastern Mississippi drainage.	Creaser & Hubbs, 1922; Hubbs & Trautman, 1937; Hubbs & Potter, 1971.
fossor	n-p	As *unicuspis*.	As *unicuspis*.
castaneus	f-p	North west and south of Mississippi drainage; other Gulf of Mexico rivers.	Hubbs & Trautman, 1937; Hubbs & Potter, 1971.
gagei	n-p	Southern Mississippi drainage; Gulf of Mexico rivers from Rio San Jacinto (Texas) to the Ochlockonee river (Florida).	As *castaneus* and Dendy & Scott, 1953.
bdellium	f-p	Ohio river system; Wabash and Upper Tennessee rivers.	As *castaneus*.
greelyi	n-p	Mountain tributaries of the Ohio river in New York State, Ohio, West Virginia and Kentucky.	As *castaneus*.
hubbsi	n-p	Mountain tributaries of the Upper Tennessee river in western Carolina and north-west Georgia.	Hubbs & Potter, 1971.
Petromyzon			
marinus	a	Varanger Fiord (Norway) and Helsingfors (Gulf of Finland) in the east; Baltic, Atlantic, and Mediterranean drainage to Jugoslavia; not Iberia. North America from west coast of Greenland to north Florida. Landlocked form in Lakes Cayuga, Ontario, Erie, Seneca and Oneida.	Creaser & Hubbs, 1922; Berg, 1931 & 1932; Zanandrea, 1958 & 1961 Tambs-Lyche, 1963; Lebedev, Spanovskaya, Savvatova, Sokolov & Tsepkin 1969; Hubbs & Potter, 1971.
		Grand Banks.	Bigelow & Schroeder, 1948.
Caspiomyzon			
wagneri	a	Caspian Sea; rivers Volga, Ural, Terek, Kura, and rivers of the Lenkoran district.	Berg, 1931, 1932 & 1948; Lebedev *et al*, 1969; Hubbs & Potter, 1971.

largest species being truly oceanic.

Of the four large anadromous species one, Caspiomyzon wagneri, is confined to the Caspian Sea in its adult phase. The other three are widely ranging as can be seen from the larval distribution. Geotria australis is found in Australia and New Zealand and in southern South America; Petromyzon marinus occurs on both sides of the North Atlantic and Entosphenus tridentatus is trans-Pacific in distribution.

Geotria has been found as an adult off South Georgia Island (Permitin, 1966; Ivanova-Berg, 1968) and Entosphenus has been trawled from depths of 380m in the Gulf of Alaska and the southern Bering Sea (Abakumov, 1964) and off the coast of Baja California (Hubbs, 1967). Scars caused by this lamprey have been reported on whales caught off Vancouver Island and Pike (1951) suggested that some of these scars were caused while the whales were further south. A more detailed list of the distribution of the species is given in Table I.

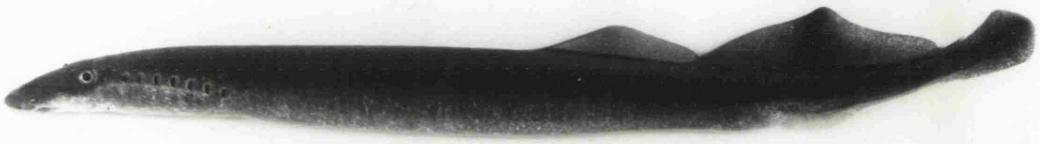

Figure 4. Lateral view of an adult river lamprey. ($x\frac{1}{2}$)

(Facing p. 12)

Figure 5. Shows the buccal disc of a mature P. marinus.

(Facing p. 12)

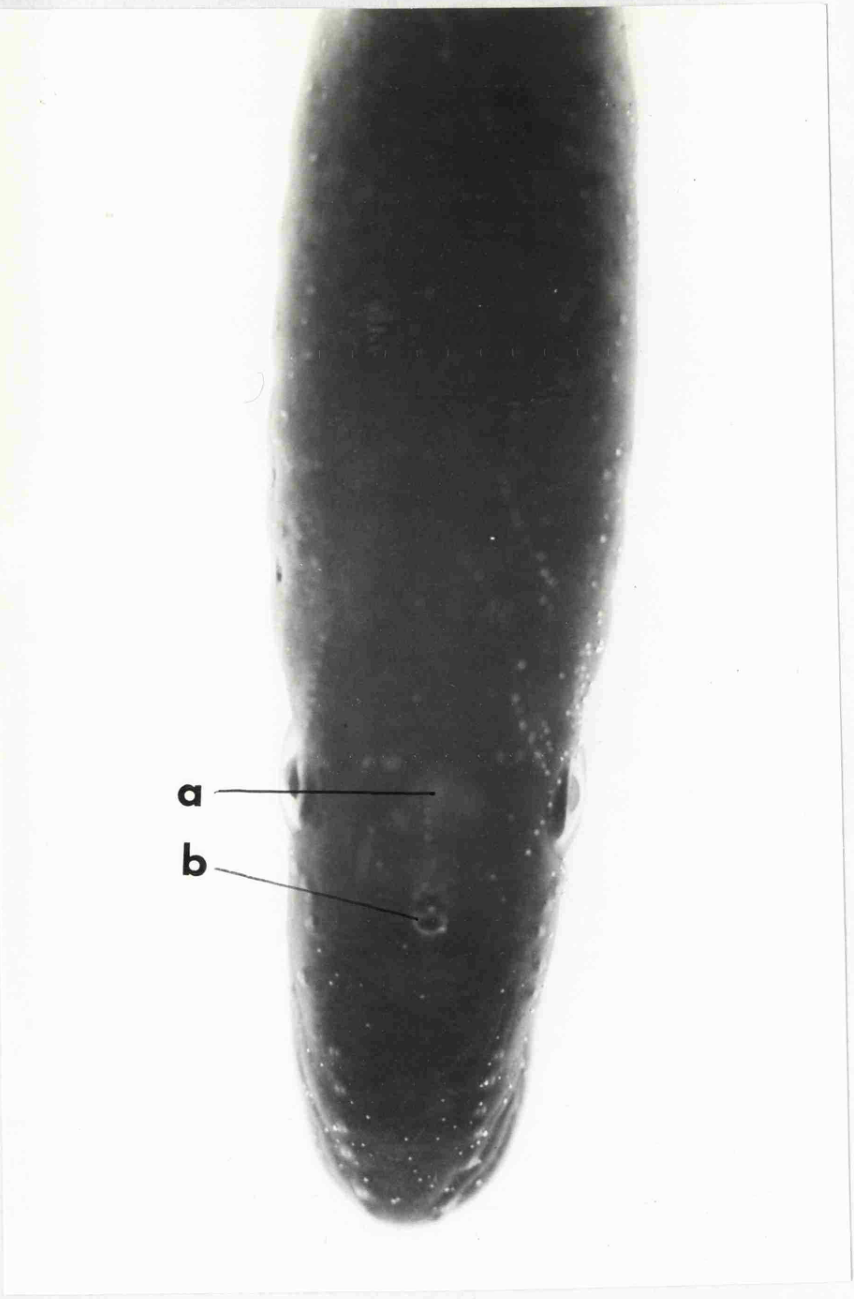

Figure 6. Dorsal view of the head of fluviatilis showing the pineal (a) and the naso-hypophysial opening (b).

(Facing p. 12)

C. General Biology

1. Appearance.

a) Adult lampreys.

Adult lampreys vary in size according to species. The largest, up to 800mm long, being the anadromous species and the smallest, down to about 80mm long,are the non-parasitic species. All are similarly proportioned. The body is eel-like, being slightly thicker dorso-ventrally than laterally, and the head, trunk and tail regions merge smoothly into one another (Fig. 4).

The head and branchial regions are together about one quarter of the total body length. The antero-ventral mouth is surrounded by a circular disc armed with horny teeth, which, with the muscular buccal funnel forms a suction apparatus (Fig. 5). The muscular tongue inside the buccal funnel is also toothed and it is used both as a piston in suction and as a rasp in feeding.

The eyes are well developed and are placed at the sides of the head. As the eyes have no external musculature, forward vision is possible only by body movement. The single naso-hypophysial opening is dorsal and just anterior to the pineal which shows through the skin on top of the head between the eyes (Fig. 6). There are seven gill pouches on each side, each with a separate circular opening.

The anterior dorsal fin is triangular and discrete except at sexual maturity. The posterior dorsal fin is continuous with the diamond-shaped caudal fin which, running forwards ventrally to the anus, forms a small ventral fin. These fins have supporting cartilagenous rays.

The smooth skin has no scales and contains abundant mucus-secreting cells. Adult lampreys are grey, brown or greenish

Figure 7. Lateral view of a planeri ammocoete to show the oral hood (a). (x 5)

(Facing p. 13)

in colour according to species, individual variation and their physiological condition. The underside is usually lighter than the dorsal surface and some species, e.g. Petromyzon marinus, are mottled.

b) The ammocoete.

The freshwater filter-feeding larva, the ammocoete, has no oral disc but there is a large upper lip called the oral hood, (Fig. 7). At the pharyngeal end of the buccal cavity is a velum, similar to that of Branchiostoma, from which a ciliated groove runs backwards through the pharynx with the groove, velum and the branchial chambers comprising the filter-feeding apparatus. The eyes are rudimentary and covered with skin. There is no anterior dorsal fin and the ammocoete usually differs from the adult in colouration being generally more darkly pigmented.

2. Life Histories.

Although the post-metamorphic life histories of the different groups of lampreys are dissimilar, the pre-metamorphic period is the same except in duration.

Lampreys produce large numbers of eggs, the non-parasitic species being the least productive. For example, the mean egg production per female of Lampetra planeri is given by Hardisty (1964) as 1,400. The anadromous species produce considerably more eggs and Vladykov (1951) gives a mean figure of 171,000 eggs for Petromyzon marinus.

The eggs hatch in two to four weeks (Hardisty, 1957; Baxter, 1954), the exact timing being dependent upon temperature. Only a small proportion (5.3% to 7.8%) of P. marinus eggs actually produce larvae (Manion, 1968). It is likely that temperature is important in determining the hatching rate of the eggs and the viability of the embryos (M cCauley,1963).The larvae lie in the gravel of the stream

bed for a further three or four weeks (Baxter, 1954) before burrowing in mudbanks. The preferred substrate for the rest of the larval life is silt with a high organic content and a small admixture of sand. Such banks are usually found at the edges of streams or in eddies. Schultz (1930) noticed that ammocoetes of Lampetra richardsoni were rarely found in mid-stream banks, presumably as these banks are too mobile, nor did he find ammocoetes in silt with growing plants, perhaps because these banks are too compacted. Baxter (1954, 1957) found that L. planeri ammocoetes occurred in streams with a gradient of between 10 and 30 feet per mile with surface currents averaging 0.5cm/sec. M. Thomas (1962) investigated ammocoetes of L. wilderi and Petromyzon marinus and placed the upper limit of current speed at 0.6 to 0.8cm/sec.

Ammocoetes burrow head first in the banks, using the oral hood as an anchor and once the photosensitive tail is under the substrate surface, they turn to lie head uppermost at a slight angle to the surface (Applegate, 1950; M.Thomas, 1962). The mudbank is left only occasionally, either at night usually during the second hour of darkness, or when the O_2 tension falls to near the lethal level (Potter, Hill & Gentleman,1970) Knowles (1941) reported larger catches of L. zanandrei ammocoetes in early morning than at midday and it is possible that they burrow more deeply later in the day.

Ammocoetes are selective filter feeders although the mechanism of selection is not clear. Manion (1967) found that the diatom Navicula represented 32.9% of the food of P. marinus ammocoetes when they were 70-72mm long although Navicula was only 7.7% of the diatom population in the bottom waters of the stream. He also found that Fragilaria was only 8.7% of the food of 33-35mm ammocoetes

even though it was 31.7% of the bottom diatom flora.

Estimates of larval life for any one species vary considerably. Lampetra fluviatilis larval life is put at 6 years by McDonald (1959), 4½ years by Hardisty and Huggins (1970) and at 3 years by Privo'lnev (1964). Petromyzon marinus has a larval life of 5, or possibly 6 years as estimated by length-frequency distribution analysis (Hardisty, 1969). Hardisty and Huggins (1970) suggested that the larval life of L. planeri is 6½ years. The larval life of Ichthyomyzon fossor was estimated using larvicides to be 3 to 4 years by Purvis (1970) and Nikolskii suggested that L. japonica is a larva for 4 years (1956).

Metamorphosis takes place in late Autumn and early Winter; external changes taking place within a few weeks, but internal changes usually taking longer (Hardisty, Potter and Sturge, 1970; Young and Cole, 1900). At metamorphosis the adult characteristics of buccal disc, teeth, eyes and anterior dorsal fin are acquired. In anadromous species the newly metamorphosed macrophthalmia becomes very silvery. Internally a new foregut replaces the larval foregut although this may never become functional in the adults of non-parasitic species. In these non-parasitic species the gonads develop rapidly.

Non-parasitic species spawn in the Spring following their metamorphosis but the parasitic species migrate down river at this time. L. fluviatilis begins to migrate in late March or April (Hardisty, Potter and Sturge, 1970), although Weissenberg (1925) found macrophthalmia in the river Elbe in May. They appear to be moving downstream during the Spring tides in the river Severn and we have taken them whilst trawling for elvers.

The length of the feeding phase is not known with certainty for any species. Mordacia mordax spends the Summer and early Autumn

after completing metamorphosis feeding in freshwater lakes, moving to the sea for a further probable 18 months feeding (Potter, Lanzing and Strahan, 1968). The smaller anadromous species probably all spend 18 months to 2 years in the sea, but there are no estimates of the duration of the marine phase of the larger anadromous lampreys.

Adult lampreys are parasitic on various fish species, feeding by means of the sucker and the mobile rasping tongue. Reports of lampreys found feeding naturally are unfortunately few and their distribution during this period is uncertain. However, adult Lampetra fluviatilis were reported in the Baltic by Benecke (1880) and by Weissenberg (1925) in the North Sea off the English coast; they were also found by Zanandrea (1959a) in the Gulf of Gaeta. It is probable that this and other small marine species stay in inshore waters as do the young adults of the larger species; Mansueti (1962) reported young P. marinus feeding on the Atlantic menhaden (Brevoortia tyrranus) in Chesapeake Bay. Large anadromous adults have been caught in oceanic waters; Entosphenus tridentatus in the Bering Sea and the Gulf of Alaska (Abakumov, 1964) and off Baja California (Hubbs, 1967) and Geotria australis have been caught near South Georgia Island (Permitin, 1966).

Scars caused by lamprey attack have been found on various marine fishes, especially salmon (Abakumov, 1960) and whales (Pike, 1951; Slijper, 1962). The freshwater parasitic species Ichthyomyzon unicuspis and I. castaneus are known to feed on at least 16 different species of fishes although one of these, Esox lucius, represents one third of the prey, (Hubley, 1961). It is possible that during the parasitic stage adult lampreys will attempt to feed on whatever they can catch.

Migration of the anadromous species into the rivers begins in late Summer and early Autumn (for Lampetra fluviatilis see Weissenberg, 1925; Hagelin & Steffner, 1958 and Evenett & Dodd, 1963). There is evidence that some anadromous species have an early migrating population ascending the rivers in Autumn, and a later population migrating in Spring (Pers. Comm. B. J. R. Taylor.). Autumn migrants probably over-winter in deep water near the spawning grounds.

The changes associated with sexual maturity and details of spawning behaviour are referred to in the Results Section (III below).

Spawning is in Spring or early Summer, and the exact time is usually dependent on water temperature (Baxter, 1954; Lohnisky, 1966; Young & Cole, 1900; Schultz, 1930; Hagelin & Steffner, 1958 and personal observation). Spawning sites are chosen primarily for their substrates, light conditions and current being secondary factors (Hagelin & Steffner, 1958). A stony or gravelly site is always chosen and a depression (the redd) is excavated by both sexes prior to spawning (Young & Cole, 1900; Schultz, 1930; Lohnisky, 1966 and McIntyre, 1969). Lohnisky (1966) and McIntyre (1969) reported that non-parasitic species spawn at depths of between 20 and 100cm, as did Young and Cole (1900), and between current speeds of 80 and 140 cm/sec.

It is unlikely that the degenerative changes of internal organs that occur during maturation could be reversed, and as far as is known, all lampreys die within a few weeks of spawning.

L. planeri and L. fluviatilis

P. marinus

Figure 8. The distribution of melanophores in the caudal fin of ammocoetes. (After McDonald, 1958)

(Facing p. 18)

D. British Lampreys

The three British lampreys are Petromyzon marinus, Lampetra fluviatilis and L. planeri.

1. Petromyzon marinus (the sea lamprey).

P. marinus is a large anadromous species with a Trans-Atlantic distribution. Land-locked races occur in several lakes of the United States of America. The ammocoetes live in sandy silt in unpolluted rivers. They are never as common as the L. fluviatilis/ L. planeri ammocoetes with which they are usually found, and from which they can be distinguished by the caudal pigmentation (Fig. 8.). Sites containing pure P. marinus ammocoete populations have very occasionally been found, and it is possible that segregation of P. marinus ammocoetes is usual, but rarely detected because of a preference for inaccessible sites. Certainly in some rivers, e.g. the Tawe, far fewer marinus ammocoetes have been found than would be expected from the size of the spawning population. Ammocoetes grow to about 150mm in length; length-frequency distributions suggest a larval period of six years (Hardisty, 1969a).

No metamorphosing or macrophthalmia stages of European marinus have been found, and so the time of metamorphosis remains uncertain, but it is likely to be similar to that of other lampreys, i.e. late Summer and early Autumn.

There is no information on the feeding phase of European marinus but because of the size of the adults the prey must be large, for example fishes such as cod, or sharks or Cetaceans.

We have caught adult marinus of up to 800mm. They are rounder in cross section than the adults of the other British lampreys, and as well as the usual secondary sexual characters the mature males

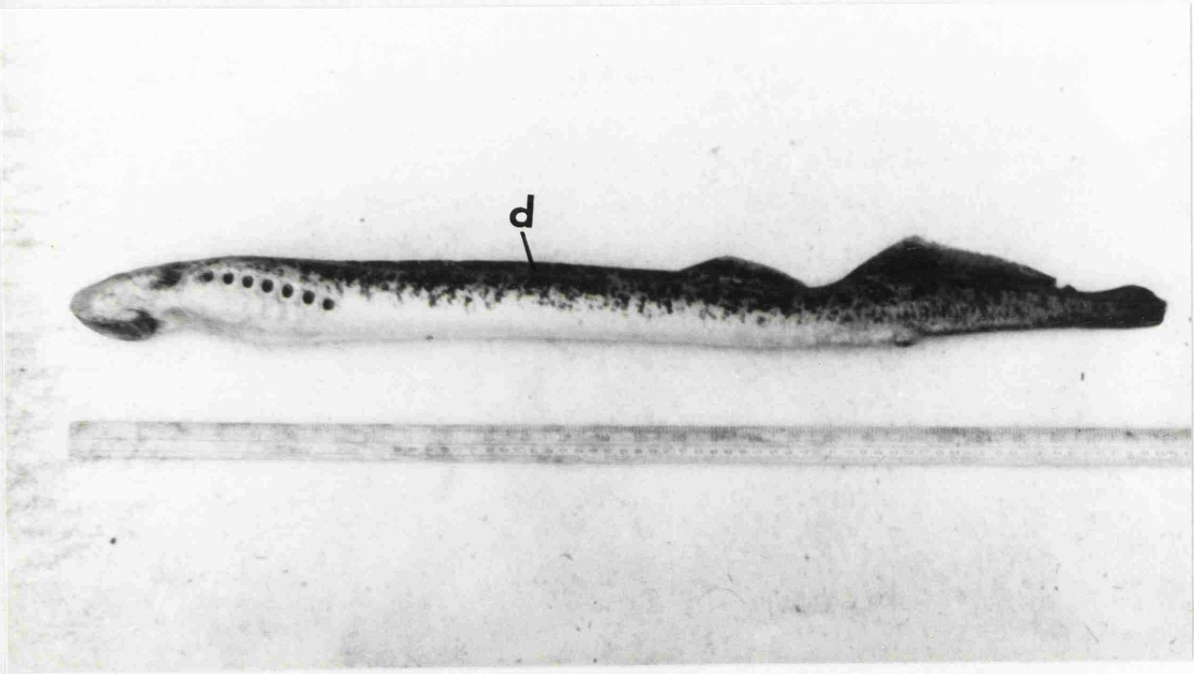

Figure 9. An adult male marinus to show the dorsal ridge (d).

(Facing p. 19)

have a pronounced 'cord-like' dorsal ridge (Fig. 9). Adult colouring is variable and ground colour may be anything from a dirty grey to a bright irridescent gold. There is an irregular pattern of dark grey, brown or black blotches over the body and fins.

Spawning adults have been found in the rivers Tawe, Teme, Tywi and Usk; usually below a weir in fast flowing reaches where the bed is of large gravel or pebbles. These adults have probably been lying under weirs or in other deep water pending sexual maturation and spawning as we have found immature adults in such places. Spawning takes place over several weeks between mid-May and the end of June depending upon water temperature. Immature adults have been caught in August at a weir on the river Severn and it is probable that at least part of the population begins its upstream migration in the Summer of the year preceding spawning.

2. Lampetra fluviatilis (the River Lamprey).

Lampetra fluviatilis is a small anadromous species with a more restricted global distribution than that of P. marinus but in Britain their distributions are similar.

The ammocoetes of fluviatilis often occur with those of marinus and almost always with those of Lampetra planeri. Some populations that are predominantly fluviatilis with only a few marinus and no planeri have been found (Hardisty, 1970; Hardisty and Huggins, 1970). The larval life of L. fluviatilis is probably 4½ years, the final year being a 'resting' year with little or no increase in length and possibly even some shrinkage (Hardisty and Huggins, 1970). Metamorphosis is at lengths between 80 and 120mm in late July and

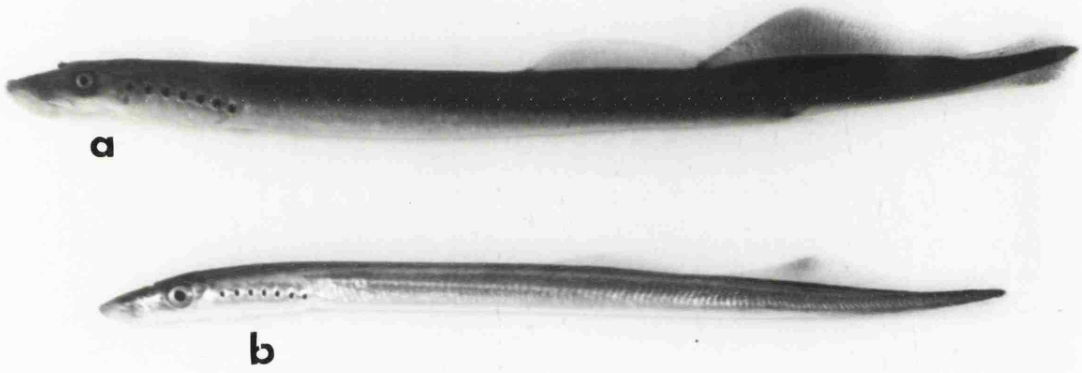

Figure 10. A comparison between an adult planeri (a) with a fluviatilis macrophthalmia (b). (Life size.)

(Facing p. 20)

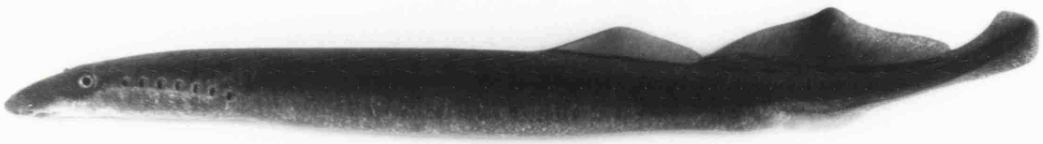

Figure 11. A ripe female fluviatilis. (x ½)

(Facing p. 20)

early August. The external changes are completed within a few weeks but the macrophthalmia probably do not migrate d ownstream until the Spring (Hardisty and Huggins, 1970; Hardisty, Potter and Sturge, 1970). Macrophthalmia have regularly been caught from a trawler in the lower Severn river in March. (Fig. 10)

Feeding is possibly triggered in the macrophthalmia by entry into salt water. In aquarium experiments, fluviatilis did not feed unless in sea water, when they attempted to feed upon dead plaice and dabs.

The majority of fluviatilis probably feeds in the sea but small lampreys of about 200mm, larger than adult planeri and smaller than fluviatilis upstream migrants, have been caught in the lower river Severn. It is possible therefore, that some fluviatilis feed in estuaries or fresh water.

Adult fluviatilis are up to 350mm in length; they are usually dark grey with a much lighter ventral surface. When ripe, most show a green or bronze colouration associated with bile breakdown products (Sterling, Meranze, Winsten and Krieger, 1967). Upstream migration begins in the Autumn but there seems to be an additional population of Spring migrants which are not morphologically differentiated. Spawning takes place in March or April, often on sites used later by marinus. (Fig. 11)

3. Lampetra planeri (the Brook Lamprey).

L. planeri is a non-parasitic derivative of L. fluviatilis and the differences in internal and external morphology between the two are a reflection of the different life histories.

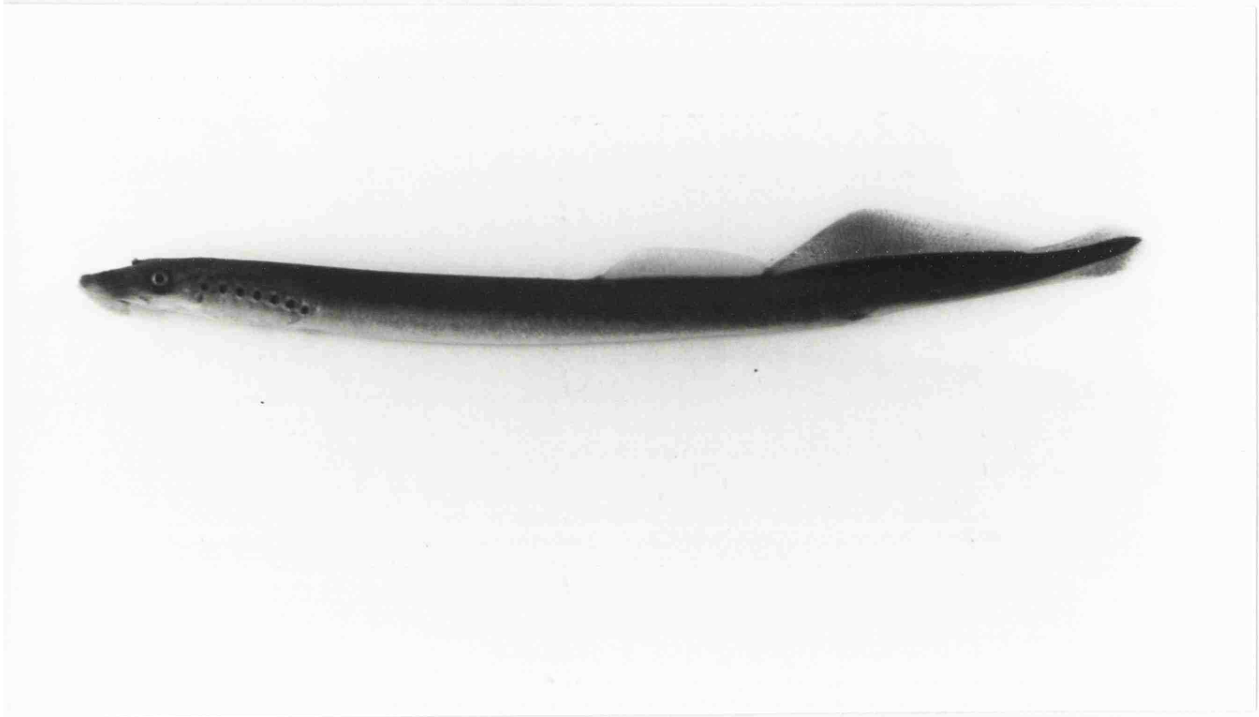

Figure 12. An adult planeri. (Life size.)

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L. planeri ammocoetes occur much higher upstream than those of the other two lampreys and pure populations are common, especially in small streams (Hardisty, 1944, 1961a). The duration of larval life in planeri is probably 5½ years, the last year being a resting period (Hardisty, 1961b).

Metamorphosis is in late Summer or early Autumn and at a larger size than in fluviatilis, being between 85 and 190mm (Hardisty, Potter and Sturge, 1970). The brown or greenish-brown fully grown adults are usually 160mm or a little less in length, although New Forest planeri are smaller, being about 100mm long when spawning. Shrinkage occurs during sexual maturation. (Fig. 12)

It is probable that planeri has been derived from fluviatilis by delayed metamorphosis, thus reducing adult life to a short non-feeding phase. This relative lengthening of the ammocoete period would account for the larger size of planeri at metamorphosis and also for the slowing down of oocyte growth during the last two years of larval life (Hardisty, 1969b). The number of oocytes produced by planeri is much smaller than that of fluviatilis (5,000 - 10,000 oocytes in planeri, 11,000 - 26,000 in fluviatilis; Hardisty, 1964). The egg number in planeri is further reduced by atresia. It is likely that the fecundity of planeri is limited by their small adult size, both by physical capacity and by the use of some of the eggs as a food reserve during the non-feeding period.

There is little difference between the ecologies of fluviatilis and planeri; ammocoetes of both are found together in the same banks, and planeri have been quite frequently observed spawning in fluviatilis redds. Attempts at 'copulation' by planeri males with small fluviatilis females have been observed but, as far as it has been possible to tell, these have been unsuccessful (Huggins and Thompson, 1970).

E. Methods of identification of ammocoetes

The identification of lamprey ammocoetes has often been problematical, especially with regard to the 'paired-species'. By 'paired-species' is meant those lampreys in which a non-parasitic form has been derived from a parasitic form and which still closely resemble each other in their ecology and their physiology (Creaser & Hubbs, 1922; Leger, 1924; Hubbs, 1925; Hubbs & Trautman, 1937; McDonald, 1958; Zanandrea, 1959bc, Hardisty, 1960a, 1961bc, 1963, & 1970; Privol'nev, 1964; Potter, 1968a & b; Potter, Lanzing and Strahan, 1968;). Many attempts have been made to differentiate the ammocoetes of Lampetra planeri and L. fluviatilis but few are useful when dealing with individuals rather than samples.

1. 'Biological differentiation'

Weissenberg (1925, 1926 & 1927) concluded that differences between planeri and fluviatilis were attributable to differing modes of adult life, as have Potter, (1968a & b) and Potter, Lanzing and Strahan (1968) in the genus Mordacia.

Hardisty (1944) found pure populations of planeri in streams below a certain size, although pure populations of fluviatilis cannot be assumed at any site (Hardisty, 1961bc & 1969a).

Length-frequency distribution analysis can be used to determine the identify of populations in some cases, the duration of larval life being 5½ years minimum in planeri and at least one year less in fluviatilis (Hardisty 1961 b; Hardisty & Huggins, 1970). A length-frequency curve should therefore show a corresponding number of peaks.

The disadvantage of these ecological methods is that they must, of course, be used on samples and/or populations and be related to the site from which the sample was derived.

2. External morphology

Numberous attempts to find morphological differences between paired-species ammocoetes have been largely unsuccessful. Weissenberg (1925) reviewed Lönberg (1893), Berg (1906), Loman (1912) and Rauther (1924) and concluded that, up to then the ammocoetes of Lampetra planeri and L. fluviatilis could not be morphologically differentiated.

McDonald (1958) distinguished among the three British ammocoetes on the bases of the shape and pigmentation of the caudal fin, and the pigmentation of the branchial area. Whilst these criteria may be reliably applied to Petromyzon marinus ammocoetes above a certain size, no external morphological characters are suitable for identifying larval fluviatilis and planeri.

Vladykov (1958) suggested pigmentation differences in the tongue precursor between the two Lampetras but this observation has yet to be confirmed.

Lohnisky (1968) suggested that there is so much individual morphological variation in L. planeri that external criteria can never be successfully applied to the genus Lampetra. However, it is possible that a deep analysis of external morphological parameters may yet yield useful results on large samples if the principles of numerical taxonomy be applied although this would at best give only a probability of identity (Mayr, 1965; Sokal, 1966; Sokal and Sneath, 1963; Smirnov, 1968; Ouelette & Qadri, 1968).

3. Internal morphology

Weissenberg (1925 & 1926) distinguished between the metamorphosing larvae of planeri and fluviatilis by means of the foregut, which is open for a period in fluviatilis but normally closed in planeri. Detailed descriptions of the changes in this region are given by Keibel (1927) and Kraentzel (1933). However, it is

possible that the foregut is open in planeri more frequently than Weissenberg (1925 & 1926) suggested (Hardisty, Potter & Sturge, 1970), especially as this characteristic is not always found in other non-parasitic species (Gage, 1928).

Attempts to identify ammocoetes using counts of mytome numbers have been more successful. Hubbs and Trautman (1937) and Raney (1941) have devised keys for the identification of ammocoetes of the genus Ichthyomyzon using this character. Cotronei (1927a & b), Vladykov (1955), Vladykov and Follett (1958) and Zanandrea (1957a) found no significant difference in the mytome numbers of the two lampreys, planeri and fluviatilis. Hardisty (1961c) found that the ranges overlapped. McDonald (1958) reported clear differences with no overlap in numbers of mytomes and although his report was perhaps overoptimistic, it is possible that some individuals at the extremes of the range may be identified using this character.

Average oocyte numbers in the larval ovary are different in planeri from those in fluviatilis, although there is overlap of the ranges of numbers (Weissenberg, 1927; Zanandrea, 1959b) and Hardisty, 1960a, 1961c, 1963 & 1964). Individual female ammocoetes above a certain low minimum size (about 40mm) can in most cases be identified by this means. Weissenberg (1925) reported and confirmed an observation by Benecke (1880) that newly metamorphosed fluviatilis have smaller oocytes than planeri at the same stage, although oocyte sizes are identical at metamorphosis. However, macrophthalmia can more easily be distinguished by external characters (Hardisty, Potter & Sturge, 1970).

Weissenberg (1927) also distinguished the ammocoetes on the basis of the size and number of testis follicles but this has not been confirmed (Hardisty, 1970).

These diagnostic characteristics can occasionally be used with certainty but seldom, if ever, in a mixed population of *planeri* and *fluviatilis*, especially for the identification of individual animals. In some circumstances individuals can be positively identified by histological or similar methods but these are applicable only to limited periods of the life history and in the case of oocyte numbers, only to the females. If the identification involves the death of the animal, this often precludes comparative physiological investigations.

F. The immune response of lampreys

Immunological competence is characterised by a series of responses to foreign materials (antigens) in which specific substances (antibodies) that combine with the antigen are produced. To be defined as immunoligcally competent, an organism must:-

i. possess gamma-globulins, some fractions of which are produced as circulating antibody.

ii. be capable of rejecting homografts, i.e. grafts of tissue from non-related animals of the same species.

iii. show delayed hypersensitivity, i.e. an allergic reaction.

iv. show either an anamnestic response to a second exposure to an antigen or to a second set homograft. (=immunological memory.)

(Finstad & Good, 1966.)

It is unlikely that any invertebrate is truly immunologically competent. Cooper (1968 & 1969) has shown homograft rejection in earthworms, and Stephens (1959) has induced a specific protective response in the moth, Galleria mellonella, to bacterial strains, but the majority of invertebrate protective mechanisms seem to be non-specific involving phagocytosis, encapsulation or intra-cellular digestion (Good & Papermaster, 1964).

Papermaster, Condie, Finstad and Good (1964) investigated the immune response of the hagfish, Polystotrema stouti, an found no evidence of immunocompetence but Hildemann and Thoenes (1969), keeping hagfish under more natural conditions, found that they produced agglutinin to sheep erythrocytes, and recognised and rejected homografts with a reduced second-set graft rejection time.

Lampreys have lymphoid tissue in the protovertebral arch or 'fat body' (= primitive bone marrow), and some lymphoid cells in the mainly epithelial protospleen (Good, Finstad, Pollara and Gabrielsen, 1966). The lampreys were also shown by these authors

to have serum gamma-globulins.

Neither Bovine Serum Albumin nor Bovine Gamma Globulin is cleared from the circulation, and bacteriophage is cleared only by some individual lampreys (Finstad and Good, 1964). During these experiments, no antibody to these three antigens was found but a definite agglutinin to killed Brucella abortus was produced which seemed specific. A second exposure to B. abortus induced a secondary, more rapid response producing more antibody. Pollara, Litman, Finstad, Howell and Good (1970) obtained high titre agglutinating sera against human 'O' blood cells.

Homografts were rejected within 42 days (Finstad & Good, 1964) with a much shorter second-set rejection time, usually five days or less. Technically perfect autografts were retained indefinitely (Finstad & Good, 1966).

Injection of Freund's complete adjuvant containing Mycobacterium tuberculosis produced necrotising lesions; administration of old tuberculin, 29 days after the initial F.C.A. injection produces an inflammatory allergic response (Finstad and Good, 1964 & 1966).

A. Cooper (1967) paralleled these investigations of lamprey adults, using ammocoetes, and obtained similar results in vivo. He also tested lymphocyte immunocompetence in vitro and found this to be greater than the whole animal response suggested; concluding that the method of presentation of antigens and their recognition is more important in Cyclostomes than in higher vertebrates.

The level of immunological competence in lampreys is low when compared with that of mammals but, nonetheless, all elements of the immune response are present.

II. MATERIALS AND METHODS

A. Introduction

1. The advantages of immunology in taxonomy

"Antibodies formed by the stimulus of a given antigen usually do not react with other antigens unless these antigens are closely related." (Ouchterlony, 1958). This specificity is the basis of all immunological tests.

Grabar and Williams (1955) stated that immunochemical reactions are of particular interest because their sensitivity and specificity are much greater than those of the vast majority of chemical or physical tests. Immunology applied to the problems of taxonomy is of special value because, as stated by Owen, Stormont and Irwin (1947), ".....the cellular antigens, without known exception, are gene controlled, the immunological techniques provide a basis for a genetic analysis of species similarities and differences even when hybridization is impossible." That blood cell antigens are genetically controlled in the Columbidae was shown by Cumley and Irwin (1944) and in chickens by Briles, McGibbon and Irwin (1950).

Many workers have used immunology as a tool to investigate the relationships of animals, plants and micro-organisms, from intraspecific and racial levels, to Orders and even Classes. Nuttall (1901) was the first to apply immunology to taxonomic work. He used whole blood as an antigen to investigate the relationships among families of Primates, among several species of Canidae, and among bovine, ovine and equine herbivores.

Boyden (1933) investigated some amphibian species, and Gemeroy (1943) used a ring test technique to differentiate among 31 species of fish. On the basis of his results, he suggested that some hitherto accepted phylogenetic relationships were not as

close as had been supposed.

Cumley and Irwin (1944) were able to correlate the occurrence of blood antigens with geographical range and therefore possible lines of phylogeny in the genus Columba. Manski, Halbert, Auerbach-Pascal and Javier (1967) have investigated phylogeny in the Classes of lower vertebrates using ocular lens protein as an antigen.

Reports of intraspecific differences in antigen systems investigated immunologically are many. The classical work of Landsteiner (1900), who investigated human agglutinins, laid the basis of blood grouping. Similar blood group systems have been found in Clupea harengus (Sindermann and Mairs, 1959) and in cattle by Owen, Stormont and Irwin (1947). Cumley and Irwin (1943) detected variations in human sera.

From these examples it can be seen that immunology has great potential in elucidating supra- and sub-specific differences. Where a single, pure, antigen is used the response is all or none; an antiserum to a related antigen either reacts with the first antigen or it does not. Most materials used in these studies, however, comprise antigen systems and the possibility of finding varying numbers of antigens, common to some species and not to others, greatly enhances the degree of sensitivity of immunological tests.

The majority of antigens are proteins and all proteins except a very few with low molecular weights are antigenic. The initial choice of material is therefore very wide. Any single material or extract is without further purification, likely to contain several antigens and thus allow the possibility of detailed investigations.

For this study a material was needed that could be obtained from whole, live animals and that could perhaps be collected from

single ammocoetes. For these reasons and others given in the next section, external mucus was the material chosen. Immunological techniques, using mucus as an antigen, seemed well suited to this purpose as other methods were either less potentially sensitive or required too much in the way of materials or equipment.

2. Selection of antigen

The choice of antigen in this study was limited both by the nature of the animals and by the exclusion of some proteins whose characteristics are unsuitable for the reasons reported below. It was hoped that a method of identification of live ammocoetes could be developed and so it was logical to use an antigen that could be gathered in usable quantities without adversely affecting the ammocoetes.

Some of the materials that have been used for biochemical taxonomy, such as ocular lens protein (Manski, Halbert, Auerbach-Pascal and Javier, 1967) and kidney and liver esterases (Nyman, 1965) are therefore unsuitable, however ideal their antigenic characteristics.

Skin tissue is a highly complex antigen system and therefore not suitable for raising antisera although small pieces of fin tissue can be taken from adults, and possibly ammocoetes, without causing severe injury. It would however, be incapacitating or even lethal to small ammocoetes if the amount of tissue necessary were removed.

Various blood proteins have frequently been used in the investigation of taxonomic relationships. In most cases it is possible to collect a useful volume of blood without killing the

the animal but this has proved impossible in lampreys. Total blood volume in ammocoetes is very small and even decapitation yields only very small quantities (less than 0.1ml from a large ammocoete). The blood of spawning adult and all lampreys to some extent, clots quickly and heparinisation followed by decapitation has been necessary to collect as much as 2.0ml from an adult fluviatilis.

If this difficulty of collection could be overcome and live animals made to yield sufficient quantities, it is possible that some blood proteins could be used. However, from the work of the authors reported below it is clear that no blood proteins so far investigated have the requisite specificity.

Electrophoresis of haemoglobins has often been used in fish taxonomy and in some species the haemoglobins are species-specific. They are specific in most Oncorhynchus species (Pacific salmon) (Tsuyuki, Roberts, Vanstone and Markert, 1965) and also in the Scorpaenidae (rockfishes) (Tsuyuki, Roberts, Lowes, Hadaway and Westrheim, 1968). One genus of the Scorpaenidae, Sebastodes, is comprised of a large number of species, most of which are morphologically very similar, but it has proved possible to distinguish among them by means of their haemoglobin electrophoretograms (Barret, Joseph and Moser, 1966). However, lampreys do not seem to possess species-specific haemoglobins. Manwell (1963) and Uthe & Tsuyuki (1967) found that ammocoetes of two species from different genera, Ichthyomyzon unicuspis and Petromyzon marinus, gave the same electrophoretic pattern while Potter and Nicol (1968) found that the pattern in Mordacia mordax was the same as that in M. praecox.

Another disadvantage of haemoglobins in taxonomy is that even when species-specific, they often show considerable intra-specific polymorphism. Some members of the Scorpaenidae show

haemoglobin polymorphism (Tsuyuki et al, 1968), as do the sprat, Sprattus sprattus, (Wilkins & Iles, 1966) and various gadoids (Sick, 1961; Frydenberg, Møller, Naevdal and Sick, 1965).

Serum transferrins, closely related to haemoglobins, show polymorphism in the carp, Cyprinus carpio, (Creyssel, Silberzahn, Richard and Manuel, 1964). Six forms of transferrin have been found in the lamprey, Petromyzon marinus, by Webster and Pollara (1969).

The herring, Clupea harengus, possesses a blood group system based on erythrocyte antigens similar to that in man (Sindermann & Mairs, 1959).

Lampreys in fact, are one of the few groups so far investigated that do not show polymorphism of the true haemoglobins. Uthe and Tsuyuki (1967) have clearly demonstrated this in Lampetra lammottei (= L. wilderi), and Potter and Nicol (1968) found no evidence of haemoglobin polymorphism in the three Australian species of lampreys.

The other feature of taxonomic importance of many haemoglobins is that they change during the life history. This haemoglobin shift is naturally commonest in fishes with a major ecological change during their life history and is probably adaptive. All lampreys so far investigated show major differences between the electrophoretic patterns of larval and adult haemoglobins, for example P. marinus, Ichthyomyzon unicuspis and L. lammottei investigated by Uthe, Roberts, Clarke and Tsuyuki (1966) and Uthe and Tsuyuki (1967); L. planeri by Adinolfi and Chieffi (1958) and Adinolfi, Chieffi and Siniscalco (1959); L. fluviatilis by Allison, Cecil, Charlwood, Gratzer, Jacobs and Snow (1960); and Mordacia mordax, M. praecox, and Geotria australis by Potter and Nicol (1968). Coho salmon, Oncorhynchus kisutch, and sockeye salmon, O. nerka, show changes

associated with their anadromous life histories (Vanstone, Roberts and Tsuyuki, 1964; Tsuyuki, Roberts, Vanstone & Markerts, 1965). Wilkins and Iles (1966) found haemoglobin changes throughout the life cycle of Clupea harengus associated with sexual maturation and even increase in length and weight.

Thus, haemoglobins are not suited to the study of taxonomic differences in lampreys. Whilst, unlike those of the more advanced vertebrates, they are not polymorphic, they are not species-specific and show major ontogenic changes.

Serum proteins have been shown to be species-specific in some turtles (Zweig, and Crenshaw, 1957) and in some species of north European Salmo by Nyman (1965). He also showed the serum proteins to be polymorphic in many other species, e.g. Perca and Abramis, as did Ridgeway, Cushing and Durall (1958) in Oncorhynchus nerka, Beckman (1965) in birds and Smithies (1955 & 1959) in man. The intra-specific variations of the serum proteins in Coregonus species (Char) are such that a characteristic individual pattern is shown by each fish (Nyman, 1965). During the same investigation ontogenic changes of the serum proteins were found to be common in pike, Esox lucius.

In Petromyzon marinus there are variations, especially in the serum albumin, associated with stages in the life history, sex differences and disease (Thomas & McCrimmon, 1964). Serum proteins therefore have the same taxonomic disadvantages as do haemoglobins.

Muscle myogens are easily obtained but only following injury or death of the animal. They are species-specific in Oncorhynchus (Tsuyuki, Roberts & Vanstone, 1965); in gadoids and some flatfish (Cowie, 1968). Viswanathan and Krishna Pillai (1956) compared

different species of fishes from California, and Lillevik and Schloemer (1961) did likewise with Great Lakes faunas. They found that the muscle myogens were species-specific, except between two closely related Sardinella species in which Viswanathan and Krishna Pillai (1956) reported equivocal results. Tsuyuki, Roberts, Lowes, Hadaway and Westrheim (1968) found that myogens in the Scorpaenidae were species-specific and polymorphic in four genera but not in the fifth genus.

Unfortunately, lamprey myogens do not appear to be species-specific, Uthe and Tsuyuki (1967) finding the same electrophoretic pattern in Ichthyomyzon unicuspis and Lampetra lammottei both as ammocoetes and adults.

None of the proteins commonly used in electrophoretic taxonomy seemed suitable therefore, as an antigen in this study.

As mentioned above, almost all antigens are proteins. All animal tissues and secretions so far tested contain protein and so the choice of antigen was not for this reason restricted. External mucus was chosen as being the only material readily available from live animals. Although it is not produced by lampreys in such quantities as in the Myxinoids (Strahan, 1959), the lampey is a slimy animal. When adult marinus are handled, this quality is readily appreciated and it was hoped that even ammocoetes, if suitably stimulated, could be made to yield enough mucus to use as an antigen.

Although other substances can be antigenic, it is probably a fair generalisation that the suitability of a material as a good antigen rests upon its protein content, non-toxicity and general 'injectionable' qualities and, especially in immunotaxonomy, its specificity.

The protein content of external fish mucuses has been found to be generally high. Wessler and Werner (1957) gave ranges of nitrogen content (by Kjeldahl analyses) from 15.2% in the cod, Gadus calliaris, to 12.7% in the ray, Raja sp., whilst Myxine mucus had a nitrogen content of 13.6% (nitrogen content of 16% is equivalent to 100% protein). Bolognani and Bolognani-Fantin (1963) reported on the mucus of the eel, Anguilla anguilla, and found a sialic acid content of 1039µg and a hexosamine content of 1171µg per 100mg of dried mucus. Ferry (1941) isolated a soluble protein from the mucus of Polistotrema stouti (hagfish), with a yield of 5% of the accompanying fibrous protein.

The other principal component of external fish mucus is comprised of various polysaccharides. These have been investigated by Enomoto and Tomiyasu (1960a & b) and Enomoto, Nagao and Tomiyasu (1961) in the loach, Misgurnus; the conger eel, Astroconger; the eel, Anguilla and the ray, Raja: by Bolognani and Bolognani-Fantin (1963) in Anguilla and by Wessler and Werner (1957) in various sea- and fresh-water fishes. It is improbable, but not impossible, that these polysaccharides and glycoproteins (mainly hexoses and hexosamines) are antigenic, as an increasing number of polysaccharides has been found to be so.

It was possible that lamprey mucus would be toxic to rabbits but fortunately this was not the case. It is likely that it is distasteful to predators and antibiotic properties for fish mucus have been suggested by Jakowska (1963). Mucus has proved to be species-specific where this has been investigated. O'Rourke (1959) and Barry and O'Rourke (1959) used paper chromatography with external fish mucus to distinguish between the pollack, Gadus

pollachius, and the coalfish, G. virens; and also between the Norway haddock, Sebastes marinus, and S. mentella, two species of the family Scorpaenidae, already noted for its taxonomic complexity. The mucuses of these four fishes are species-specific.

O'Rourke (1961) found serum proteins in external fish mucuses and suggested that this might account for the specificity of the mucus and possibly provide a mechanism for the recognition of prey by specific fish predators. Enomoto and Tomiyasu (1960a & b) and Enomoto, Nagao and Tomiyasu (1961) found slight differences in the amino acids and hexose contents of the mucus from various species of fishes.

A general advantage of the use of mucus as an antigen is its ubiquity. "Mucus is a secretion that occurs almost universally in the animal kingdom" (Jakowska, 1963). Almost all fishes secrete external mucus and all animals have mucoids in some form in their bodies. There was the possibility therefore, that the choice of mucus as an antigen might enable an investigation of the relationships of the Cyclostomes with other groups.

B. Collection of lampreys and field work.

1. Ammocoetes

Lamprey ammocoetes used in this study were usually caught after they had been stunned with an electric shocker (Marine Electrics Cybertronic Mark 10 Mini Trawl). The plate electrode was pushed into the mud of a bank likely to contain ammocoetes, and the net electrode used at distances of up to six feet. Banks were fished working upstream to avoid clouding the water. The ammocoetes emerged from the mud in a semi-stunned condition and were netted as they revived and began to swim away. A few collections were made by scooping up mud likely to contain ammocoetes with a net and the mud was sorted through on the bank.

Collections were usually made during the Summer although one collection was made during a very cold period in January but it was only partly successful. It is possible that in cold weather, the ammocoetes burrow more deeply and are too stunned by the electric shocks to reach the surface of the mud.

Ammocoetes were placed into plastic boxes containing ice-cooled water for travelling and on arrival were transferred to tanks in a cool, constant temperature aquarium with aeration. The temperature in the aquarium was usually $8^{o}C$ except during the spawning season when it was raised to $11^{o}C$ to hasten sexual maturation of metamorphosed adults. The sand substrate in the tanks grew some algae but no additional food was given. Ammocoetes kept thus survive well and will metamorphose.

Lampetra planeri ammocoetes were collected from the Ober Water and Blackwater river in the New Forest, the river Wylye near Warminster (Wiltshire), the Afon Gwydderig (a small tributary of the river Tywi in South Wales), and the river Honddu (a tributary

of the river Usk near Brecon in Wales). No adult L. fluviatilis has been found in these streams and length-frequency distribution analyses of ammocoete populations suggest pure L. planeri populations (Hardisty, 1961).

L. fluviatilis ammocoetes were caught at Bransford Bridge on the river Teme from sites which were shown to contain mainly L. fluviatilis with a small number of Petromyzon marinus ammocoetes (Hardisty & Huggins, 1970).

P. marinus ammocoetes were collected from various sites on the river Tawe (Devon) and the river Teme at Bransford Bridge. As stated above, large ammocoetes of this species can be easily identified by their caudal pigmentation.

2. Adults

Most of the adult L. fluviatilis used were caught during their upstream migration from October to March in a trap set below Tewkesbury weir on the river Severn by the River Board Bailiff. The remainder were caught on their spawning grounds at Ashford Carbonel on the river Teme and below Shrewsbury weir on the river Severn. They were kept in wire cages in a pond at the University.

Adult L. planeri and P. marinus were caught on their spawning grounds after the observation and filming of their spawning behaviour. Adult L. planeri, all sexually mature, were netted in March or April on gravel beds in the New Forest streams; the river Blackwater at Queen's Bower and Warwick Slade, the Ober Water at Boldreford Bridge and the Highland Water in Burley Lodge Walk. They were transported in the same way as the ammocoetes and were kept, with others that had attained sexual maturity in the aquarium in a glass walled tank

for observation and filming of spawning behaviour.

Adult P. marinus were caught in June and July at or near their spawning grounds but unlike L. planeri many sexually immature adults were found. P. marinus adults are not easily caught; their size makes netting difficult, they are wary, and the immature individuals are especially powerful when compared with the spawning adults. The best method of catching them was by hand. After a careful approach, the lamprey was grasped quickly and very firmly with both hands, one around the middle and one around the gill region, and thrown into a large net held by an assistant. Aqualungs and wet-suits were usually neccessary, especially where, as in the Tawe, immature and non-spawning individuals collected in deep pools below a weir which prevented their further upstream progress.

The day was usually spent filming their behaviour. The animals were caught just before return to the laboratories to minimise the duration of their confinement. They were transported in large plastic dustbins, two or three to each bin, and the water was kept cool by polythene bags filled with ice and frequent additions of loose ice. The water was usually aerated during travel. Unlike ammocoetes and adult Lampetra, adult P. marinus did not generally travel well. Immature individuals survived best but losses of up to 50% were unfortunately frequent. It was found possible to revive seemingly dead individuals by massage of the gills and heart region with the animal under cold water. This resuscitation was occasionally successful up to 30mins after arrival at the laboratories.

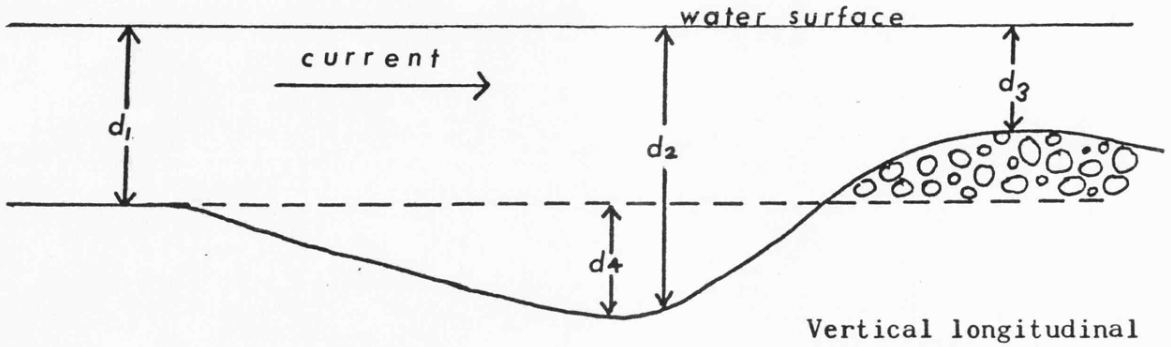
P. marinus adults have been found spawning at: several sites on the river Tawe and its tributary the river Mole in Devon; the river Teme below the weirs at Powick and Ashford Carbonel; the river Usk near Llangynidr and the river Tywi above and below Llangadog.

Most of the P. marinus adults were released in the South Building pond at the University, where in spite of rather warm water they survived well. The rest were kept in large galvanised tanks in the aquarium but here only a few could be accommodated and frequent water changes were needed. The advantages of keeping individuals in the pond were rather outweighed by the difficulty of recapturing them. Adult marinus learn quickly and will rapidly and consistently swim to the other side of the pond when one stands on the edge. Although their sight when sexually mature appears extremely poor and their eyes look opaque, they seem to have no difficulty in avoiding a net. What sensory apparatus is used is in doubt because they will paradoxically swim directly into a fixed obstacle or even out of the water and up the river bank when they are disturbed.

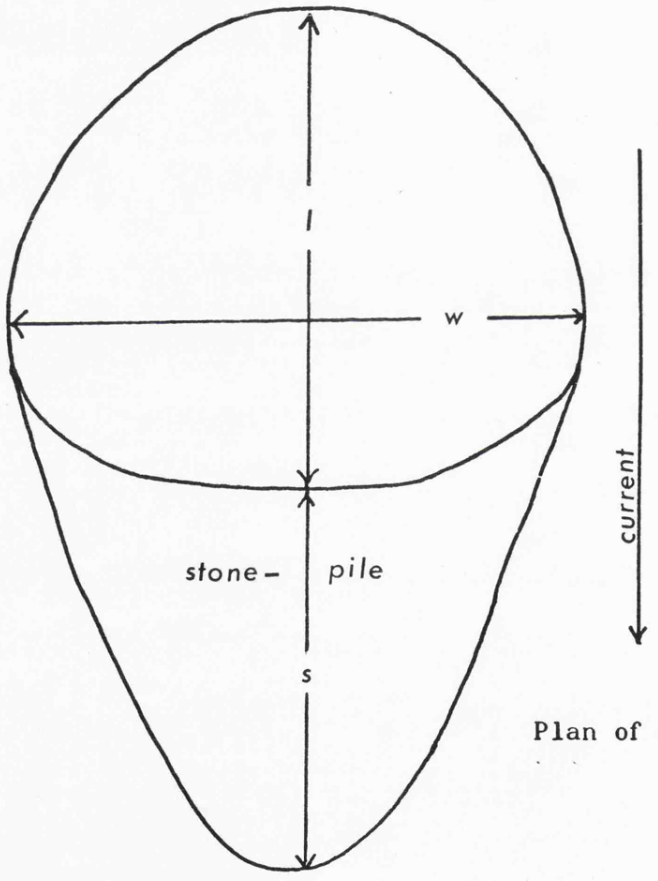
3. Filming of spawning behaviour.

During the course of this work, the opportunity was taken to assist with the filming of spawning behaviour. Although lampreys always seem to spawn when the sun is out, on many occasions water clarity was too poor to provide suitable filming conditions. Added to this difficulty, lampreys only spawn on a few days each year and so the chance of them spawning when water and weather conditions permit filming is low.

A Bolex H16 Reflex 16mm cine camera was used for all river filming with Kodachrome II (B-winding) Daylight reversal film stock. All film was exposed at 24 frames per second. To expose the film, the camera was fitted with an f/1.4 10mm Switar RX wide angle lens and placed in a Bolex Underwater Housing. It was usually necessary, even for filming planeri, to use a wet-suit and snorkel to lie on the river bed with the animals. The presence of a cameraman and



Vertical longitudinal section of redd.



Plan of redd.

Figure 13. Redd diagrams to show position of measurements referred to in the text.

(Facing p. 41)

an assistant appeared not to disturb any of the lampreys when they were redd building or spawning. P. marinus could even be gently handled underwater without alarming them.

For the indoor filming of planeri spawning sequences, the film stock was Kodachrome IIA (B-winding) and illumination was provided by a Eumig quartz/iodine cine light. The high light intensity enabled the lens aperture to be set at f/22 giving a large depth of focus. The behaviour pattern did not appear to be disturbed by this high light intensity.

P. marinus and L. planeri were the principal subjects for filming as on those occasions when L. fluviatilis were found spawning, the water was always too turbid to allow adequate exposure.

4. Redd measurements

The following measurements were made of the redds of L. planeri and P. marinus (Fig. 13)

i. The distance from the water surface to the bed of the river just in front of the redd (= d_1)

ii. The distance from the water surface to the bottom of the redd (= d_2)

iii. The distance from the water surface to the top of the stone pile behind the redd (= d_3)

iv. The depth of the redd below the stream bed level (= d_4)

v. The greatest downstream length (= l)

vi. The greatest cross stream width of the redd (= w)

vii. The current speed on the surface of the water.

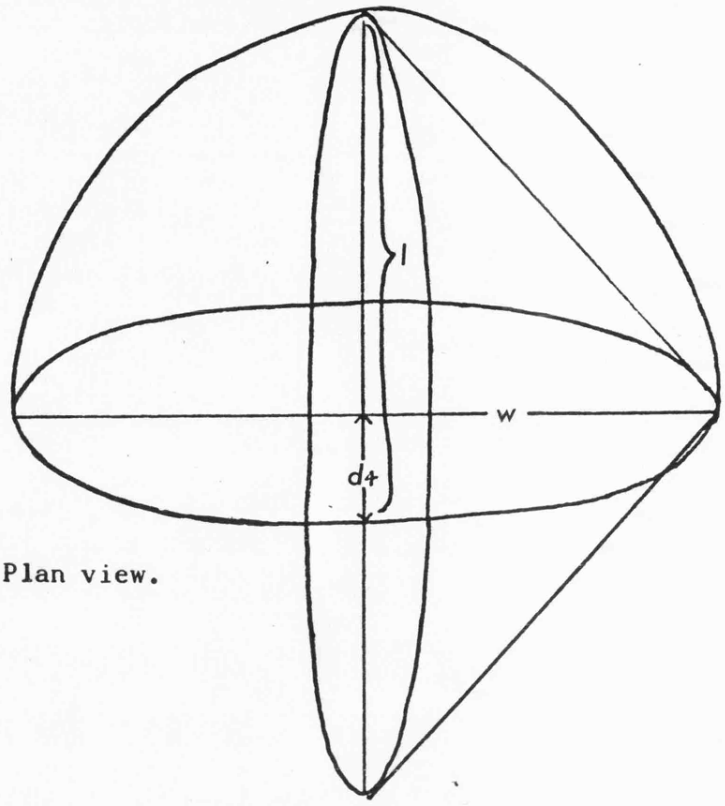
viii. The length of the stone pile downstream from the redd (= s).

Linear redd measurements were taken with a metre rule. Current speeds were measured by timing the passage of a neutrally buoyant bottle near the surface over a measured metre, three or more times for each redd. Care was taken to ensure that the observers did not interfere with the current flow. Samples of stone piles and river bed material were collected for subsequent analysis.

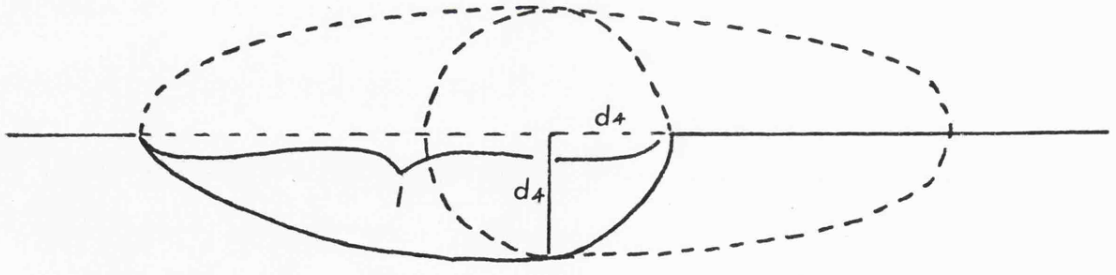
Eighteen planeri redds in the Ober Water of the New Forest were measured in early April 1970. The number of animals in each redd was recorded and the population of one redd was caught for subsequent weighing and measurement. It was felt that, as this area of the Forest is very popular during Easter when spawning generally occurs, and the lamprey population is therefore subjected to severe predation by small boys, removal of the contents of more than one redd would be a further detrimental interference with the natural population.

Eleven marinus redds in the river Tywi, just below Llangadog, were measured in early July 1970. No lampreys were then present but from the condition of the redds it was apparent that spawning had only recently ceased. This conclusion can be justified by the fact that in these spawning grounds algae quickly cover stones that have been recently disturbed, while fresh redds have the algae-free surfaces of stones exposed. After a longer period of time, depending on current conditions in the river, redds also fill with river bed gravel. After redd measurements had been taken, samples of the river bed and stone pile were collected.

Stone samples were weighed, both as individual stones and as total samples, and sample volumes were measured by displacement. The total approximate volume of each redd was found by the equation,



Plan view.



Longitudinal vertical section.

Figure 14. A diagram of a stylised redd to show the geometrical approximations whose volumes were calculated to give an estimate of redd volume.

(Facing p. 43)

$$\frac{\pi w(d_4)^2}{6} + \frac{\pi(d_4)(L - d_4)(w)}{12} \quad cc. \qquad \text{Eq. 1.}$$

This equation has been derived as follows. The volume of the cap of depth d_4, of a solid ellipse with circular cross section =

$$\frac{\pi(w/2)(L/2)(d_4)^2}{3(L/2)^2} \cdot \left\{3(L/2) - (d_4)\right\} \qquad \text{Eq. 2.}$$

However, this assumes that the cross section of the redd parallel to current flow is as illustrated in Fig. 14. In fact, the downstream cross section is nearer to Fig. 13 and Eq. 2 would not give a good estimate of the volume. However, the depth of the redd (d_4) was usually equal to, and always close to, the distance from the deepest point to the downstream end of the redd. Consequently, Eq. 2 simplifies to:-

$$\frac{\pi w(d_4)^2}{3} \qquad \text{Eq. 3.}$$

This gives twice the volume of the redd downstream from a plane perpendicular to the current flow at the deepest part of the redd. If half of this volume is added to half of the volume of a cone (height = w/2) with an elliptical base (d_4 to L-d_4), the total volume of the redd is estimated as in Equation 1.

This method of estimating redd volume introduces a small error because of the assumption of a regular geometrical form. However, it is the closest approximation possible without having to collect a logistically impossible number of dimensions for an iteritive calculation of the volume by a computer - an exercise of doubtful worth because each redd is a reflection of an unique set of circumstances.

C. Collection of antigens

Enomoto & Tomiyasu (1960a) collected external mucus by dropping their fishes (loach) alive into ethanol and scraping the resulting precipitate from the skin. As the lampreys in the current study have been in short supply and only for a limited period of time each year, a less drastic technique had to be developed.

Lampreys used for mucus collection were anaesthetised with 1 part of tricaine methanesulphonate (Sandoz M.S.222) to 1000 parts (w/v) of distilled water. Thorson (1959), investigating the effects of M.S.222 on P. marinus, found that this dilution gave rapid anaesthetisation with no fatalities even after prolonged exposure. It took about 10 min to anaesthetise adult P. marinus, about 3 min for adult L. fluviatilis and considerably less for ammocoetes and L. planeri adults. The duration of unconsciousness depended upon the length of exposure to M.S.222, but animals were usually removed from the anaesthetic on cessation of respiratory movements which normally restarted within 15min. When the mucuses from live anaesthetised and freshly killed animals were compared by gel diffusion the patterns obtained with any one antiserum were the same.

Adult L. fluviatilis, caught during upstream migration, were usually available in large numbers between October and March and initial collections of mucus were made from these animals. The anaesthetised lamprey was blotted dry and placed on moistened paper towels in a waxed dish. Drops of distilled water were pipetted on to the skin and scraped to and fro with a spatula. Mucus was taken up by the water and the resultant 'blobs' removed by pipette. Mucus was produced partly in response to dehydration and the initial

blobs were clear and only slightly viscous while later blobs from the same animal were opalescent and much more viscous. This process of scraping was continued until spontaneous gill movements began, at which time the animals were transferred to an aerated recovery tank. When fully recovered the animals were returned to holding cages in the pond, or to tanks in the aquarium. No animal was scraped more often than every other day.

Except in specific cases, in which the mucus was used to test for sex differences &c., mucus from several individuals was pooled. It was then diluted with distilled water to five times its original volume and centrifuged at 3000rpm for 10min to throw down algal cells, epidermal tissue and any other debris. The supernatant was freeze dried for storage until needed.

Dry weight yields averaged about 1.0mg per animal (fluviatilis) scraped during mid season months but yields decreased with increasing sexual maturity. It was possible to scrape about 15 animals a day in mid-season but from the end of February until the beginning of April the number of fluviatilis available declined to none. Losses of material due to mechanical failures of the freeze-drier were sometimes as heavy as 30% and so the 1967-1968 season total of 0.560gm dry weight represents mucus from about 700 animals.

After the first season, 10% ethanol replaced distilled water during scraping and by this means yields were increased by about one third. Care was taken to avoid getting ethanol into the gill openings, eyes, naso-hypophysial opening and mouth. Scraping with ethanol provoked inflammation of the skin but this soon passed and the animals rarely appeared to suffer permanent damage. Presumably the irritant and dehydrating effect of ethanol produced both the inflammation and the increased secretion of mucus.

P. marinus adults were too large to scrape in the manner described above. They were suspended by the head, well protected with damp foam plastic and paper towels, from a clamp. The body behind the gills was then washed down with 10% ethanol. Unlike L. fluviatilis, P. marinus tended to secrete more mucus as they approached sexual maturity and scraping was often unneccessary. When they were suspended and flushed with ethanol, quite concentrated mucus sometimes dripped from the tail. Yields from marinus varied with size and sexual maturity but on average was about 15mg dry weight at each collection. Animals were always in short supply and, as they were generally collected at spawning time from spawning reaches they died soon after capture. 580mg dry weight were collected from May to July 1969.

Adult L. planeri and all ammocoetes yielded very poorly. The following methods of stimulating mucus production were applied to the skin:-

i. mild electric shock
ii. 3% sodium chloride solution
iii. 0.1N hydrochloric acid
iv. Ethanol in various concentrations up to 30% Beyond this concentration the skin dehydrated quickly, severely and sustained permanent damage. Intracoelomic injections of pilocarpine (Jakowska, 1962) made up to 0.2% in lamprey saline were also tried. Neither this nor any of the other methods improved on the yields obtained with 15% ethanol in distilled water. Yields from large ammocoetes (140mm or larger) were up to 0.3mg dry weight per animal.

Where mucus from individuals was required it was collected with the addition of as little ethanol as possible and used raw, i.e. without centrifugation or freeze drying. Quantities of mucus from individuals were so small in comparison with pooled collections that the usual processing would have resulted in proportionately much

heavier losses. Samples of mucus from the eel, Anguilla anguilla; plaice, Pleuronectes platessa; salmon smolt, Salmo salar and a small Gadus sp. were collected from freshly killed specimens and used raw.

The protein content of lamprey mucus was estimated by total nitrogen content determined by micro-Kjeldahl analysis.

When more powerful complex antigens were required, lamprey skin extracts were used. Pieces of skin (dermis and epidermis) or whole skins from smaller lampreys were cut up and ground with sharp sand and a little distilled water. This mixture was then exposed to ultrasonic agitation for 10min, ground again and then replaced in the ultrasonic agitator for a further 15min. Lamprey skin is very tough and the skins of even small ammocoetes remained whole after this treatment but sufficient of the cell contents were released to enable the use of the supernatant as a powerful antigen.

Antigen solutions, other than those used for immunisation, were protected with sodium merthiolate (= Thimerosal) 1 part to 10,000 antigen solution (w/v) and stored in the deep freeze.

Muscle samples for chromatography were removed from the sides of large lampreys. Ammocoetes were decapitated, skinned, gutted and had the notochord removed and the remaining material used in muscle preparations.

D. The preparation of antisera.

The antigenicity of proteins differs; for example, Bovine Serum Albumin is not as immunogenic in rabbits as is ovalbumin. Because the antigenicity of external lamprey mucus was unknown and because only small quantities of antigen were available the rabbits were immunised with an antigen/Freund's Complete Adjuvant mixture.

Adjuvants cause a prolonged and enhanced immune response. Freund's Complete Adjuvant (= FCA), which is the most commonly used adjuvant, is a mixture of a light mineral oil (often paraffin oil), an emulsifier (usually Arlacel A) and dead mycobacteria. Its effect is threefold. The water-in-oil emulsion formed when soluble antigen in saline is thoroughly mixed with FCA is relatively insoluble in vivo; the contained antigen is therefore slowly released giving a prolonged stimulation. Secondly, the constituents of the adjuvant usually cause an inflammatory granuloma at the injection site, associated with large numbers of macrophages, cells which are possibly important in the immune response. Lastly, mycobacteria cause an enhanced antibody production. The reasons for this are not clear but it is known that stimulation with dead mycobacteria causes production of large numbers of macrophages all over the body. (Freund, Jefferson, Hough, Sommer and Pisani, 1948; Steiner, Langer & Schatz, 1960 and Kind, 1970.)

The initial antisera were raised in six New Zealand White rabbits, each weighing about 2.5kg. Into each of these, one ml of a 1:1 FCA/antigen solution was injected subcutaneously spread among up to six sites on the shoulder. The antigen solution was 5% w/v freeze-dried fluviatilis mucus in normal saline (about 25mg protein per ml.).

The rabbits were bled from the lateral ear vein after six weeks, which yielded 20ml of whole blood per rabbit, and thereafter they were bled weekly. Antibody was first detected by a ring test in sera from the second bleeding. Eleven weeks after the initial injection, the antibody titre began to fall and the rabbits were given a 'booster' dose of 0.5ml of 1% (w/v) antigen in saline intravenously. Bleeding was continued but it was found necessary to repeat the booster dose every second week as recommended by Campbell, Garvey, Cremer and Sussdorf(1964). Because of ear scarification during the first nine months, 20ml blood samples were removed by cardiac puncture weekly thereafter. All rabbits were subsequently bled in this way.

This first series of rabbits was last bled 18 months after the initial immunisation and although the antibody titre was still high, specificity had diminished because of repeated challenges.

The blood was collected in clean tubes and left at $4^{o}C$ overnight for clot separation and contraction. It was unnecessary to centrifuge the whole blood as serum collected in this way was clear. The sera from individual rabbits varied in titre and specificity. All 'good' sera were pooled for subsequent use; this is the serum referred to as 'A' in the results. 'Good' sera are those which on plate double gel diffusion against homologous antigen yielded three or more precipitin lines and that were not noticeably haemolysed.

The second series of rabbits (two Californian and one New Zealand White) was injected with a 5% w/v solution of marinus mucus subcutaneously in FCA as described above. Antibody was found one month after the injection, boosters being given 6 and 7 months after the initial injection. The serum from each rabbit was kept separately, and these are referred to as M6, M7 and M8.

The third series was three New Zealand White rabbits which were injected with a 1:1 mixture of planeri mucus and FCA subcutaeously. Planeri mucus was available in minute quantities only and was not freeze dried but used raw; the protein content of the antigen injected was not known. These sera are referred to as P2, P3 and P4.

A further six Californian rabbits were injected subcutaeously with 5% (w/v) fluviatilis freeze dried mucus mixed 1:1 with FCA (1ml of the mixture spread among six sites on the shoulder). The sera were desgnated F17, F18, F19, F20, F21 and F22.

Finally, four Californian rabbits were injected with marinus mucus in FCA using a similar dosage and the sera were designated M11, M13, M14 and M23.

Five batches of antisera were therefore available; two against fluviatilis mucus, the first of which was pooled from six rabbits and the second consisting of sera from three individuals; two anti-marinus serum batches from three and four rabbits respectively with sera from individual animals kept separately; and finally anti-planeri sera from three individual rabbits. Where sera were not pooled, differences in response from individuals were marked and persistent.

Antisera were kept in 15ml aliquots in plastic containers in the deep freeze until needed. Sera in current use were kept at $4^{o}C$.

E, Immunodiffusion

1. The 'Ring' test

The 'ring' or interfacial test is usually used quantitatively to establish the equivalence point of an antigen/antibody system. In this study it has been used as a quick test to establish the presence or absence of particular antibodies in a serum and to determine the range of concentrations of antigen most suitable for the gel diffusion tests that followed. The principle of this test is that a line of precipitate develops at the antigen/antiserum interface if the concentrations of the antigen and antibody are at or near the equivalence point. Above or below these optimum concentrations the precipitate dissolves as it forms in the excess of antigen or antibody.

0.4ml of antiserum was put into each of a series of 1 x 5cm flat-bottomed glass tubes and an equal quantity of antigen solution layered on the top, care being taken that mixing of the two solutions did not occur (Campbell, Garvey, Cremer and Sussdorf, 1964). The tubes were placed in a Perspex rack for ease of viewing. In a positive test the precipitate developed at the antigen/antiserum interface within a few minutes.

In determining optimum antigen concentrations, serial dilutions from 0.01% (w/v antigen in normal saline) to 0.1% in 0.01% steps and from 0.1% to 1.0% in 0.1% steps were used. Dilutions of from 0.1% to 0.6% (= 0.5 to 3.0mg protein/ml) gave positive results.

2. Diffusion in gels

Bechhold (1905) was the first to observe precipitation rings in gels resulting from the antibody/antigen reaction, although his results were overlooked for some time. Standardised techniques

of immunodiffusion were not established until the 1940's, when Oudin (1946) reported a simple method of diffusion in tubes. Subsequent developments of diffusion-in-gel techniques were reviewed by Grabar (1957) and Ouchterlony (1958).

The principle of diffusion in gels is that the reactants diffuse towards each other through the gel, (or in some cases one reactant diffuses into a gel containing the other, Ouchterlony, 1948) and a visible precipitate forms where the reactants meet at their equivalence point. Most antigen/antibody precipitates are soluble in one or both reagents if in excess and as Ouchterlony (1949b) stated, "One can therefore visualise the process as a migration of two critical concentrations against each other, the precipitate forming where these meet....." The position of the precipitate- the expression of the point of balance between the reactants, is a function of the initial concentrations of antibody and antigen, provided that these are above a certain minimum threshold, and of the rates of diffusion of the reagents. In multiple antigen/antibody systems, many of the constituent reagents will have differing initial concentrations and rates of diffusion and so a series of precipitin lines will form.

In this study, double diffusion in plates was used (Ouchterlony, 1949a-c, 1950 & 1953). A petri dish is partly filled with agar. Wells, cut in the solidified gel, are filled with antigen or antibody which then diffuse towards each other. This method establishes the minimum number of reacting systems and also allows a qualitative comparison of antigens. Identity, partial identity and non-identity of antigens each give typical precipitin patterns in a simple gel diffusion system.

The initial appearance of the lines is the same, whatever

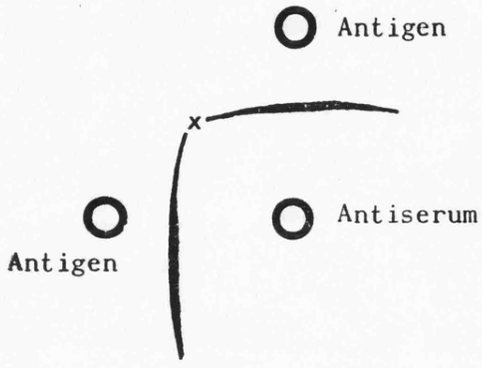


Fig. 15a.

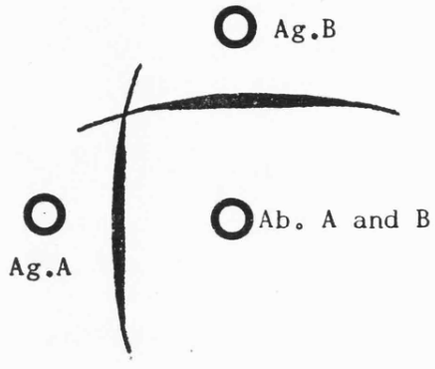


Fig. 15b.

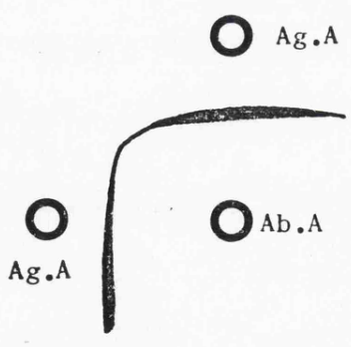


Fig. 15c.

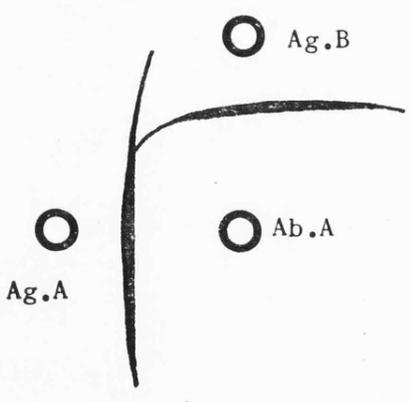


Fig. 15d.

Figure 15. Precipitin patterns in gel diffusion plates showing various relationships among antigens. (Ag. = antigen; Ab. = antiserum.)

(Facing p. 53)

the relationship between the antigens (Fig. 15a). As the lines grow laterally on further diffusion during incubation, they eventually meet at point x. At this point the possibility of interaction between the lines arises giving inhibition, deviation, or partial or complete fusion. The simplest case is where there is no interference because there is no serological relationship between the antigens compared (Fig. 15b). Where there is serological identity there will be complete deviation and fusion to give the pattern shown in Fig. 15c. Relationship between the antigens, but not identity gives rise to 'spur formation' (Fig. 15d) where there is deviation, inhibition and partial fusion (the precipitate of B is partially deviated and inhibited). This pattern is often the result of antigen A, comprised of all those antigens in B and some others being reacted against an antiserum to A.

The line of precipitate has a sharp edge on the side of the reagent not in excess and a rather blurred spreading outline on the side of the reagent that is in excess (Grabar, 1957).

Systems of double diffusion have disadvantages; bands may be superimposed and the precipitates of several antigens appear as one; single bands may appear double, especially where the excess of one reagent is very great; and under some circumstances, the typical patterns of identity &c. may be formed in other ways (Wilson & Pringle, 1954 & 1955).

Immunoelectrophoresis, where gel electrophoresis of the antigen is followed by double diffusion, overcomes these difficulties to a great extent. The increased resolving power of this technique is due to the extra 'dimension' involved, that is, the electrophoretic mobilities of the antigen proteins.

The main disadvantage of immunoelectrophoresis is that the concentration of antigen at the source of diffusion is lowered by the spreading effect of the preceding electrophoresis and greater concentrations of antigen may be needed. The following authors deal with various aspects of immunoelectrophoresis; Grabar & Williams (1953 & 1955), Grabar,(1957),Martin, Scheidegger, Grabar & Williams (1954), Scheidegger (1957) and Ouchterlony (1958).

i. Double diffusion technique

1% Bacto (Difco) agar was made up by the method given by Campbell, Garvey, Cremer & Sussdorf (1964) except that trypan blue was added to give a concentration of 0.5ml/gm agar, as the 1.0ml/gm agar suggested by Campbell et al was found to give too intense a colouration.

Small plastic petri dishes (5cm diam.) with tightly fitting lids were each partly filled with 6ml of melted agar mixture; this gave a depth of gel of about 4mm. When the gel had set, one central and six peripheral wells were cut by means of a template and hole punch attached to a vacuum line. Moistened filter papers were put into the lids which were replaced and the plates stored at $4^{o}C$ until needed. For use, each well was filled with about 0.05ml of the reagent to be tested; the antigen solutions at a concentration of 0.55% w/v in saline and the antisera used undiluted. The lids were replaced and the dishes left in a humid atmosphere at room temperature for 48 hours when the results were recorded.

ii. Immunoelectrophoresis

The method was basically that of Scheidegger (1955) which was a micromethod on microscope slides requiring smaller amounts

of antigen and antibody than the original method of Grabar and Williams (1953). Using a Shandon slide tray and gel cutter, eight microscope slides could be set up simultaneously with up to two holes and two troughs in the gel on each slide.

The barbital buffer used throughout was 1 litre of N/10 sodium barbital and 300ml of N/10 hydrochloric acid made up to 2 litres with distilled water giving a pH of 8.2.

The agar was a 1% solution of Bacto agar in the barbital buffer with sodium merthiolate added to give a concentration of 1% w/v. The agar was made up in batches of 50ml aliquots which were stored at 4^{o}C until needed, at which time they were melted in a boiling water bath.

Standard (3in. x 1in.) microscope slides cleaned with acetone and dried, were placed in the slide tray which had been moistened with a few drops of distilled water and placed on a levelling table. 50ml of melted agar was poured onto the slides on the tray, over which a 'spreader' was then passed to ensure an even thickness of gel. The gel was left to set for about 10min and then placed in a refrigerator for a further 10min to harden.

A Shandon cutter was used to cut two troughs and a central hole in each slide. The waste gel from the troughs was removed with a small knife and the gel from the holes was sucked out with a sawn-off hypodermic needle fitted with a pipette teat. An Agla microsyringe was used to fill the central hole with antigen and the tray placed in the electrophoresis tank. The buffer used in the tank was the barbital buffer given above. Filter paper wicks moistened in the buffer were placed along the edge of the slide tray touching the gel and the other side dipping into the buffer.

All runs were at a constant current of 60mA, the initial voltages being between 350 and 400volts, falling to about 250volts at the end of the 40min electrophoresis. This gave an initial voltage of between 40 and 50volts/cm.

After the electrophoresis, antiserum was pipetted into the troughs and the plates placed in a humidity cabinet at room temperature for 24hours. Unless otherwise recorded, all antigens were 0.55% solutions w/v in normal saline, and antisera were used undiluted.

After the slides had been cut from the tray, they were photographed, lit indirectly from below by a high intensity lamp fitted with a green filter. Ilford HP3 or HP4 35mm film was used in a Zeiss Contarex camera fitted with a bellow extension and an f/2 Planar lens. The effective aperture used with the bellows extended was f/24 requiring a 1sec. exposure. The film was developed in Ilford F.F. Contrast developer (diluted 1:4 with water), at 20^{o}C for 2¼min.

F. Immunofluorescence techniques

Coons and his collaborators were the first to use antibodies labelled with fluorescent compounds (Coons, Creech & Jones, 1941; Coons, Creech, Jones and Berliner, 1942). Since then this technique has been widely applied, especially for the detection of microbial or tissue antigens in sections and in the investigation of cellular antibody production. The techniques have been reviewed by Nairn (1969).

Three main modifications of Coon's original technique are most used; a) direct antigen detection, where specific fluorescent antibody is combined with its antigen; b) indirect antigen detection where antigen is combined with specific antibody and labelled anti-gamma globulin is applied; and c) antibody detection using labelled antigen.

The fluorochromes used have varied and early workers used isocyanates of polynuclear aromatic hydrocarbons (Coons et al, 1941; Coons et al, 1942; Weller & Coons, 1954), fluorescein isocyanate becoming the most popular because of its intense fluorescence (Goldman & Carver, 1957). However, as isocyanates are unstable and require the use of phosgene in their preparation, they have now been largely superceded by the more stable isothiocyanates, especially those of fluorescein and rhodamine B (Riggs, Seiwald, Burckhalter, Downs & Metcalf, 1958).

In this study, fluorescent antisera were prepared in the hope of developing a quick and easy way of identifying external mucus smears by the direct antigen method.

Two antisera were labelled; the first, a 'dummy run', used

'B' serum (i.e. antiserum from the 'A' series of anti-*fluviatilis* rabbits that was originally either somewhat haemolysed or not very specific) and the second run used M7 anti-*marinus* serum.

Conjugation was preceded by gamma-globulin isolation by ammonium sulphate precipitation. 50ml of serum was titrated with saturated ammonium sulphate solution, added in small quantities and the precipitate redissolved by stirring. After about 20ml. of ammonium sulphate has been added in this way, the precipitate will no longer redissolve, at which time sufficient ammonium sulphate is added to make up the total added to 25ml. The suspension was brought to pH 7.8 with 2N sodium hydroxide solution and stirred for a further three hours, when it was then centrifuged at 14000g for 30min. The supernatant was discarded and the precipitate was redissolved in 50ml of normal saline.

The precipitation and resolution was repeated twice more and the final precipitate was dissolved in 25ml of borate-buffered saline. The solution was dialysed against borate-buffered saline until the dialysate was free from sulphate ions, detected by adding to a small aliquot (acidified with a drop of dilute hydrochloric acid) a few drops of 2% barium chloride solution.

The protein content was estimated by the Biuret test (Campbell, Garvey, Cremer & Sussdorf, 1964) and was found to be about 1.5mg/ml. The volume of the gamma globulin solution was measured and then transferred to a chilled centrifuge tube. Fluorescein isothiocyanate Celite (FIC) was placed on the surface of the solution (0.5mg FIC per mg. protein). The tube was centrifuged under refrigeration taking about 15min to reach 1400g and then maintained at this level for 5min. The sedimented FIC was suspended with a stirring rod without foaming being induced and then transferred to a 30ml beaker, the centrifuge tube being placed in an ice bath. The pH of the

suspension was raised to 8.4 by the dropwise addition of borate buffer from a Pasteur pipette (pH being monitored with a pH meter). This adjusted suspension was returned to the chilled centrifuge tube and stirred gently for 1hr, foaming being avoided.

The suspension was then dialysed at $4^{o}C$ against eight 4-litre changes of saline adjusted to pH 7.8 with borate buffer. The dialysis was continued for three days, by which time the dialysate was visually free of yellow-green colour. The conjugate was then clarified by centrifugation, merthiolate added(1:10000 w/v) and stored in a refrigerator, (Campbell, Garvey, Cremer & Sussdorf, 1964).

Gamma-globulin solutions and conjugates were tested for antibody content by immunoelectrophoresis.

G Isolation of specific antibodies

As most of the antigens in the external mucus were common to all three lampreys, no serum was wholly species-specific. An attempt was therefore made to separate the species-specific antibodies from those common to other species.

If antiserum containing antibodies to A, B and C is mixed with antigen A, the antibody should react with the antigen, leaving free anti-B and anti-C antibodies. If the anti-B, anti-C containing serum is removed, the anti-A antibodies can be dissociated from the antigen-antibody complex to give specific anti-A antibody.

With soluble antigens, such as those of the external mucus, it is usual to adsorb the antigen onto the surface of an insoluble, inert or non-related 'filler', e.g. red blood cells, cellulose derivatives &c. In this study, glass beads were used, the adsorption and elution of the antibody achieved by the method given in Campbell, Garvey, Cremer and Sussdorf (1964), outlined below.

Glass beads (100-500μ, Minnesota Mining and Manufacturing Company, St. Paul, Minnesota, U.S.A.) were washed in concentrated nitric acid and then thoroughly rinsed in triple distilled water. The beads were used to fill a 2 x 25cm chromatography column. Triple distilled water was sucked upwards through the column and the volume needed to fill the interstices of the beads recorded. Roughly twice this volume (= 60ml) of a 0.2% w/v solution of freeze dried marinus mucus in 0.85% saline was poured down through the column, displacing the water. The outflow was stopped just before the surface of the antigen solution had reached the top of the beads. The column containing the antigen solution was kept at 4^{o}C overnight.

The column was washed through with 2l of saline and then 20ml

of P2 anti-*planeri* serum, diluted 1:2 with saline, was run into the column which was again left overnight at 4°C.

3l of saline was passed through the column to wash out the serum, the first 20ml being collected, leaving the adsorbed antibody behind in the column. This antibody was eluted from the column with glycine-hydrochloride buffer at pH 3.0 (glycine 41.8gm; conc. hydrochloric acid 8.3ml; sodium chloride 8.5gm; made up to 1l with distilled water). The eluate was collected and brought to pH 7.0 with 0.1N sodium hydroxide solution.

The adsorbed serum was placed in dialysis tubing as was the eluate and each of their volumes was reduced to 10ml by placing the tubes in contact with polyethylene-glycol-6000 (= 'Carbowax') for as long as was necessary (ca. 3h). The resulting concentrates were dialysed for 48h against saline. The activity of the serum (containing anti-*planeri* and anti-*fluviatilis* antibodies) and the eluted antibody (specific anti-*marinus* antibody) were tested by immunoelectrophoresis.

The isolation of anti-*marinus* antibodies placed a strain on *marinus* antigen supplies but because the identity of *marinus* is not nearly so problematical as that of the others, it was felt worthwhile to risk an exploratory run using up the existing stocks.

Antigens from *planeri* and *fluviatilis* were only obtained at great expense of time and effort and were in very short supply. Consequently it was deemed unwise to hazard these scarce reagents on the single run that would be possible with the quantities of mucus available. Such a run would have to be successful if the products of six months' scraping were not to be wasted. It seemed that such a 'once and for all' test should be based on experience

of more than one method of antibody isolation. Another method of specific antibody isolation has been used successfully in several runs with human IgG and rabbit anti-human IgG antibodies, using a slight modification of the method described by Porath, Axen and Ernback (1967). This uses agarose beads as an inert filler and it seems likely that this method would be equally applicable to lamprey mucus antigens and their antibodies.

200mg of Sepharose 2B (Pharmacia, Uppsala, Sweden), represented by 14ml of the suspension supplied, was diluted with 10ml of distilled water and the agarose beads resuspended. The suspension was poured into a 50ml beaker containing a magnetic stirrer slug and a pH meter electrode. All subsequent steps, in which cyanogen bromide was used, were performed in a fume cupboard.

10ml of a 50mg/ml solution of cyanogen bromide in distilled water was added to the suspension. 2N sodium hydroxide solution was added dropwise during vigorous stirring, until the pH took 5min to recover from 11.5 to 11.0. The Sepharose was then washed in a sintered glass funnel attached to a vacuum line, with 10 volumes of distilled water and 10 volumes of 0.1M sodium bicarbonate solution, both at $4^{o}C$.

Protein solution at $4^{o}C$ (150mg in 5ml of borate buffer with pH at 8.5) was mixed with the Sepharose. As 5ml or less were used, it was not necessary to stir because the Sepharose contained this volume. The protein/Sepharose mixture was left at $4^{o}C$ for 16h.

The borate buffers used were made up thus: 6.2g boric acid and 7.15g potassium chloride were made up to 1l with distilled water and the pH adjusted to 8.5 with 0.1N sodium hydroxide; and for borate buffered saline, sodium chloride was added at 5.84g/l.

Hypodermic syringe barrels with glass wool plugs in the nozzles were used as columns. The Sepharose was packed into the syringe and washed for 5min with 0.05M ethanolamine to inactivate any residual activity. The columns were then washed with about 10 volumes of borate buffered saline, 10 volumes of 0.85% saline, 10 volumes of 0.1M acetic acid and finally 10 volumes of borate buffered saline.

Antibody adsorption and elution was performed at $4^{o}C$ with continuous spectrophotometric monitoring using an L.K.B. 8300A Uvicord II (L.K.B. Produckter A.B., Stockholm).

The column was washed with borate buffer until the baseline on the Uvicord was stable. Undiluted antiserum was added to the column at the rate of 1ml of antiserum to each ml of packed Sepharose. The antiserum was washed through with borate buffer, those fractions giving a light transmission of 70% or less on elution being collected. Washing was continued until transmission returned to the base line (= 100%), when the antibody was eluted with 1M propionic acid (pH 2.5). Eluate showing a transmission of 70% or less was collected and the pH adjusted to 7.5 by dialysis against borate buffer. The column was washed with borate buffered saline until the eluate allowed 100% transmission.

Adsorbed serum and eluted antibody were reduced to their original volumes with polyethylene glycol 6000 as described above. Their activities and specificities were tested by immunoelectrophoresis and they were then dialysed against distilled water and freeze dried for storage.

A column when adequately washed with borate buffered saline and then borate buffer can be used for a further three times but the last run tended to show a fall in yield.

H Thin layer chromatography

When lampreys were unavailable in sufficient numbers for mucus collection, during the early part of this study, the amino acids of lamprey muscle were tentatively explored by thin layer chromatography.

150mg samples of individual lamprey muscle, from ammocoetes and adult male and female fluviatilis were hydrolysed with 0.5ml of 6N hydrochloric acid in sealed ampoules at 110°C for 24h. After cooling, the contents of the opened ampoule were dried under vacuum in a boiling water bath. The dried residue was dissolved in 2.5ml distilled water giving a hydrolysate concentration of ca. 2% w/v. The pH was adjusted with 0.1N sodium hydroxide if necessary.

35g of silica gel (Kieselgur G, Merck) made up in 70ml of distilled water was spread over five 20 x 20cm plates to give a thickness of ca. 250μ. When dry, the plates were activated at 100°C for 30min.

Spots of hydrolysed protein solution were applied with a micro-pipette in serial concentrations of up to x8, near the lower edge of the plate.

Good one-dimensional separations were obtained using n-butanol/ethanol/distilled water in the ratios 4:1:5 and also by methanol/benzene (1:9) but the most satisfactory solvent combination was n-butanol/acetic acid/distilled water (150:37:63 w/w).

Two dimensional chromatograms were obtained using n-butanol/acetic acid/distilled water (4:1:5) in the first direction and phenol/distilled water (75:25) in the second direction.

Spots were developed with ninhydrin, phosphomolybdic acid

or polychromatic ninhydrin (Moffat & Lytle, 1959), this last proving the most useful.

When mucus was available, some chromatograms of dialysed, hydrolysed mucus yielded no results, doubtless due to the inevitably low concentration of the mucus. This line of investigation was not pursued because the mucus could not be spared.

III RESULTS

A Observations on the spawning of lampreys

While collecting material for this and other research in the Group, the spawning behaviour of P. marinus and L. planeri has been observed. Although L. fluviatilis have been found on spawning grounds several times, spawning has been seen only once and water conditions were such that close observation was impossible.

Spawning occurs in discrete regions of the headwaters of river systems at sites which have the correct substrate, water speed and depth. All three British lampreys migrate upstream until the find the right conditions or until further upstream migration is prevented by a weir or natural obstacle such as extensive shoals. They lie in deep water among stones below the impediment until they are ripe when they move to the nearest suitable site downstream.

All of the spawning sites of marinus and fluviatilis are remote from Bath and entail lengthy journeys. L. planeri sites are closer and hence most of the observations relate to this animal. Furthermore, planeri have been induced to spawn in an aquarium tank after the provision of a suitable current.

The observation of spawning entails firstly the location of a site on a river, often by the sighting of redds following that year's spawning and secondly by visiting the site in the following year when conditions are exactly right. Spawning lasts for only a few days each year and the timing depends upon the maturation of the animals coinciding with a suitable water temperature and light conditions so it is not surprising that these observations are limited.

1 The spawning of L. planeri

Brook lampreys lie under stones in the vicinity of the spawning site until they are ripe and water conditions are suitable. When the water temperature reaches 11°C, usually in the late morning around April 10th, adults can be seen swimming over a suitable substrate with gravel and stones (see redd measurement data), the interstices of which are filled with sand. The site chosen is often just downstream from a shallower region in which the surface is rippled. There is a large stone or small branch quite often just in front of the redd, and this or the shallower water perhaps produces the 'desired' water turbulence conditions for successful spawning. A single redd has been found to contain up to 200 animals, although this is an exceptionally high number brought about by an impassable weir just above the redd. 10 occupants in a redd is more like the usual number.

Individuals of both sexes participate in the redd building. An animal sucks on to a stone and vigorously swims until the stone is dislodged when the animal tends to be carried downstream by the current. It then drops the stone and returns for another. This process in continued for some hours until all of the stones have been removed and the floor of the redd consists of a layer of fine sand. By now, the redd is quite conspicuous, being much lighter in colour than the surrounding river bed with its algal coating. If stones are too large to be moved by a single lamprey they can sometimes be moved by two or more animals pulling together. This coincident pulling is progressively more likely as fewer stones remain in the redd and it is not suggested that there is deliberate collaboration.

Although redd building has been observed in overcast conditions, the events next to be described have only been seen during sunshine, possibly because the actual mating process needs visual cues. In late afternoon (ca. 4.00p.m.) as the sun drops the animals disperse and return on the following day if they are not spent. If they are spent, they drift downstream and die.

The female swims to the front of the redd and fastens on to a stone and tries to move it. If it can be moved there is often a reversion to redd building, although if there is a male close by, a fruitless attempt at pairing may occur with both animals drifting downstream. If the stone is immobile, the female arches her back in the region of the fifth gill opening and the body becomes rigid with the tail slightly upcurved. At this stage she waves her body from side to side, usually with sufficient vigour to stir up a cloud of sand from the floor of the redd.

This appears to be a key stimulus for the male who rapidly sucks on to the back of the head of the female. Behavioural releasers are discussed by Schleidt (1962 & 1964). Often at this stage the female relaxes her grip on the stone and the manoeuvre aborts with both participants drifting downstream. If the female remains attached to the stone, the male coils his tail around the right side of the body of the female with the papilla of the male opposed to the genital opening of the female. Then follows a vigorous shaking of both animals with, one assumes, the coincident release of eggs and milt. This process lasts for at most 2secs, after which the female releases her hold on the stone and the animals drift downstream whilst disengaging. The eggs are presumably quickly fertilised and since they are sticky when released they adhere to sand grains in the floor of the redd. The females, and less often the males, fan their tails

vigorously, sending a cloud of sand downstream in the water current. This cloud of sand includes eggs, weighted down with adhering sand grains. When the sand and weighted eggs settle into the interstices of stones downstream from the redd, the eggs are protected from predatory fishes such as eels and Cottus which are often found in or near redds during spawning.

Quite often two or three males will simultaneously couple with one female, all apparently successfully completing the mating.

On occasions, although the male fastens on to the head of the female, his attempt to coil around the female is not successful. This apparently does not prevent both animals from reaching a climax since both, attached only at the head, follow the writhing of a normal pairing for the same duration. This has twice been filmed and it is a typical fixed action pattern which has to be completed once initiated (fixed action patterns are described by Eibl-Eibesfeldt, 1970).

The entire sequence of events appears to be closely co-ordinated by behavioural reflexes, as might be expected, and a high fertilisation rate is likely.

Lohnisky (1966) described the spawning of planeri, as did Schultz (1930). Lohnisky states that 'the male followed the female swimming about the nest at all times, and as soon as the female adhered, the male approached from behind and attached himself....., coiling of the male around the female following immediately' Lohnisky mentions only a few cases where pairing was preceded by the gravel-clearing type of tail lashing of the female. In no case during our observations of planeri or

marinus did a male persistently follow a female, and all attempts at pairing were preceded by tail lashing by the female. Further, this tail shaking behaviour seemed to be the key stimulus for the whole subsequent mating pattern. Males showed no interest in females that were not tail-lashing but would approach and attach to, and even attempt to mate with males that were showing the key stimulus.

The fact that males did not appear to follow the females as recorded by Lohnisky (1966) in our aquarium observations may have been due to the abnormal situation, although it has not often been observed in the field.

Young and Cole (1900) described the spawning of another brook lamprey, L. wilderi, but only very briefly.

2 The spawning of P. marinus

The spawning of the sea lamprey follows the same basic pattern described above but it is necessarily on a much larger scale. Mature, but unripe marinus first appear in the spawning reaches in late May but they are probably present in lower reaches of the rivers from the previous Autumn. Factors influencing the timing of this arrival on the spawning grounds are water temperature and the volume of water in the rivers. Sexual maturation, although intrinsically regulated, is probably hastened by rising temperatures but the greatest upstream movement takes place when the rivers are high after a period of heavy rain.

The changes associated with sexual maturity are progressive and are not all fully expressed until the animals are ripe and ready for spawning. The females become turgid with eggs and have a swollen cloaca and in both sexes the dorsal fins become continuous. The males have a prominent urinogenital papilla

and a cord-like ridge develops along the back. Degenerative changes are concurrent; the teeth become blunt and some drop out; the eyes degenerate which is surprising if visual clues are important; the gut becomes non-functional and the liver fills with bile breakdown products. The general condition of the animal deteriorates and it becomes inactive except in those aspects of behaviour associated with spawning. Fungal infection of wounds, probably by Saprolegnia, becomes general, while this is only an occasional condition in immature adults. A few ripe marinus, taken from an Ulcerated Dermal Necrosis infected river showed ulcers very similar to those produced in salmon by this disease although, as far as is known, there are no reports of this disease in lampreys. In comparison, planeri at spawning are in very good condition with symptoms of degenerative changes being minor. Spawning fluviatilis are intermediate in physical condition, although it is not uncommon for the snouts of the animals to be damaged and infected with fungus and the eyes are often becoming opaque.

P. marinus swim upstream until they reach a barrier such as a weir under which they lie in deep water until they are sexually ripe. When the river is exceptionally high the lampreys may be able to surmount a normally impassable weir and then continue upstream for several miles before spawning. This happened in the river Tawe in Devon in 1968 when the animals were seen spawning at Lapford, several miles upstream from their usual site.

When they are held by a barrier they drop downstream to a suitable spawning site when they are ripe. It is possible that later upstream migrants are chemically attracted to the spawning

grounds by the ripe adults already there. P. marinus have been found on their spawning grounds between late May and July although a warm Spring may advance the breeding season by a week or two and a few spent individuals may be found as late as August.

All of the marinus spawning grounds discovered have been in the clear, fast flowing waters of large rivers. Data reported under redd measurements give an indication of the preferred conditions. A drift survey of a part of the river Tywi revealed a few scattered redds over about 2 miles of apparently identical river. There is no weir here to bar further upstream progress. It is possible that the population is so small that many apparently ideal sites are left unused.

It is noticeable during marinus spawning and redd building how important stone size can be. The animals cannot move stones above an upper weight limit. The largest stone found in a stone pile weighed 2.3kg in air and the average adult marinus weight in air is 730g. Under water, the effective weight differences are much greater since the marinus will be almost neutrally buoyant and the stones must be moved as a result of the animal's mechanical purchase on the water. The upper limit in stone size is weight and the lower limit is the surface area of the stone which can just be gripped by the sucker. The smallest stones found in a marinus stonepile were 7cm in diameter (the largest were 30cm.).

The behaviour pattern during redd building and pairing is similar to that described for planeri, except that there is generally only one of each sex in a redd (exceptionally three animals) and the process is more slowly executed. The tail fanning of the female again is apparently the releaser for the male, who attaches to the body about midway along and then

nuzzles his way along to the head, maintaining continuous contact with the female. Abortive couplings seem to be less frequent in marinus than they are in planeri, perhaps because the animals are less vulnerable to current in the redd.

When the animals are spent they drift downstream and die.

3. The spawning of L. fluviatilis

As reported above, this has been observed once only and then unsatisfactorily. Our visits to the spawning sites have generally been either a day or two too early or too late, or if the animals were in the redds, conditions such as light have not been correct for spawning to occur. In spite of there being no description of their spawning behaviour to offer, some general conclusions can be drawn.

River lampreys appear to spawn on sites close to those on which marinus will spawn later in the year, although they select adjacent stretches where river sorting has left smaller stones on the bed of the river. The critical temperature seems to be 11°C as in planeri. On almost every occasion when fluviatilis has been found in redds, a few planeri have been found in or near the redds, sometimes spawning, as reported by Huggins and Thompson (1970). As many as a dozen female fluviatilis have been seen in one redd with only one male, but this has been when most of the animals were spent and it is unlikely to represent the situation when they are actually spawning.

Because of the temperature dependence of the animals, a wave of spawning moves northwards over the country. New Forest planeri are generally the first to spawn, followed by planeri in streams around Somerset and later by those in Welsh rivers. The same pattern is probably followed by fluviatilis. Marinus

spawning is generally first observed in the river Tawe in Devon, closely followed by Welsh and Shropshire sites, although it tends to last for a longer period (perhaps up to a fortnight) although this is a subjective impression.

Other descriptions of spawning behaviour have been given by Hagelin and Steffner (1958) for fluviatilis, and by Applegate (1950) for the land-locked P. marinus in the Great Lakes.

4 Artificial fertilisation of lamprey eggs

All three British lampreys have been successfully stripped of eggs and milt in the laboratory to produce embryos. Viable hybrids between planeri and fluviatilis have also been produced in this way although it is not known whether they will metamorphose because of the long ammocoete life. These hybrids are being tended as part of the programme of another researcher and will doubtless be the subject of a report in the future.

Stripping in all three animals is similar, so the operation will be described for fluviatilis only, followed by brief comments on the others later.

Two clean enamel trays (ca 30 x 20 x 5cm deep) containing about 1cm depth of distilled water are placed on a bench. The animals for stripping are selected for ripeness. The females have a bloated dorsal fin suffused with blood and a swollen cloaca. The body is swollen with eggs and if she is really ripe some eggs may be extruded when the animal is handled. At least two such animals are isolated in an aquarium tank.

The males are ripe when their urinogenital papilla protrudes and the whole body has a dark bronze colouration. Milt may be ejected when the animals are handled. At least two are placed in a separate aquarium tank.

The female is caught and held firmly by the head and the gill region. A wet cloth will facilitate the gripping. The right hand squeezes the animal tightly behind the gill region and the hands are drawn apart, maintaining the squeeze. This action may have to be repeated several times before eggs are extruded, especially if the animal is not quite ripe. When the eggs

are extruded, it may require several movements to expel them all. The last few eggs may be accompanied by blood stained peritoneal fluid and since this may adversely affect fertilisation, it is better to leave the last few eggs in the female.

The appearence of the eggs when extruded give an indication of the likelihood of fertilisation. Suitable eggs are a pale cream colour and if they are sluiced about by tilting the tray they will rapidly form a monolayer and adhere to the bottom. They appear not to be self-adhesive. If the eggs are likely to be infertile, they are brownish in colour and adhere only weakly if at all. In this case the second female is stripped into the next tray.

Although Kille (1960) suggested that lamprey eggs can be fertilised only during the first 50 sec after extrusion, this has not been found to be the case. It has happened that selected males could not be stripped of milt on occasions, no matter how ripe they had appeared to be and more males have had to be selected from the stock tanks. On occasions, as long as four minutes has elapsed between the extrusion of the eggs and their coverage by milt. This may be an important observation as will be seen from the discussion.

The male is held over the tray of ripe eggs and stripped of milt using the same technique as for the female. The tail of the male is much more active than that of the female and it tends to curl around the right hand of the operator so care has to be taken to point the papilla towards the eggs. If the male is ripe, the milt is easily extruded, care being taken to avoid the release of peritoneal fluid as in the female. The second

male is promptly stripped over the eggs. All animals are returned to an aquarium tank until it is convenient to kill them.

Once the milt is in the tray with the eggs, the water is sluiced from end to end in order to distribute the sperm. The tray is then left for one or two minutes, after which the water is decanted and replaced, again to a depth of about 1cm. This is left for 15min and again similarly replaced. At this stage the tray is placed in a constant temperature room at 15°C, i.e. the approximate stream temperature.

After about 1½ days cleavages can be seen in the eggs and they lose their adhesion to the tray. Infertile eggs should be removed if possible since they rapidly become covered in fungal hyphae which trap the hatched ammocoetes later. After about a fortnight the ammocoetes hatch and become free swimming. Until this stage the water should be changed every two days, but when the larvae are swimming it has been found preferable to arrange for a continuous flow change of water. The ammocoetes swim freely, absorbing their yolk sacs for about the next ten days. As soon as the yolk has been resorbed the hitherto transparent animals become pigmented and burrow, so fine silty mud should be provided.

Using this routine there have been few failures and it is suggested that provided the animals are ripe, fertilisation will occur.

The stripping of planeri is similarly achieved but they must be pinched between the thumb and forefinger of the left hand,

and stripped by pinching the body between the ball of the right thumb and the top joint of the right forefinger. Their small size makes directional squirting of the milt almost impossible and much of it ends up on the fingers. As long as they are promptly rinsed in the water in the tray, this is of no consequence.

The stripping of marinus follows a similar pattern to that of fluviatilis but because they are so powerful, it is helpful to have assistance in holding the animals. Larger dishes are desirable because the abundant eggs will not readily form a monolayer in the trays described.

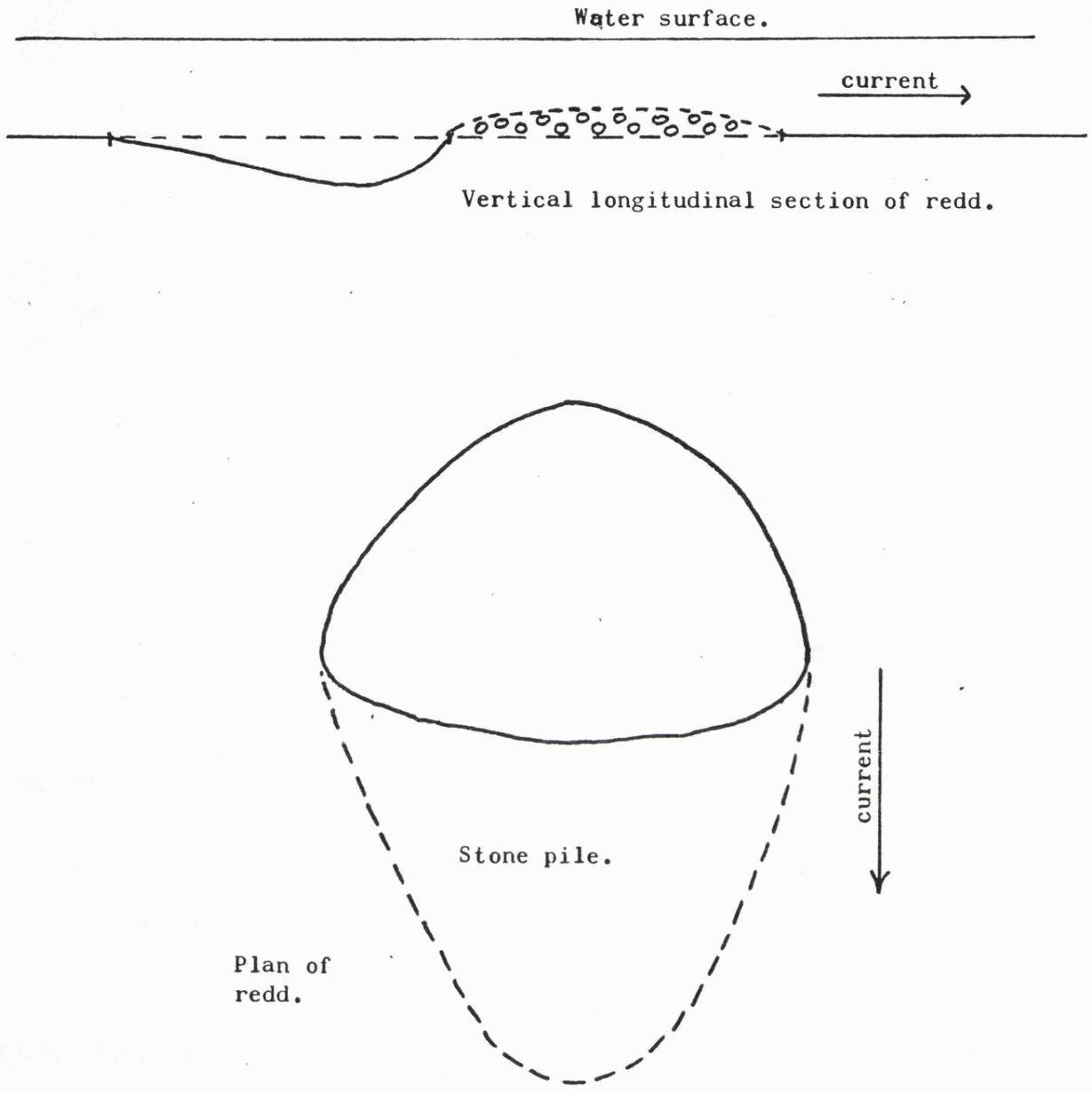


Figure 16. Diagram of the average P. marinus redd.
(Scale = x 0.05)

(Facing p. 79)

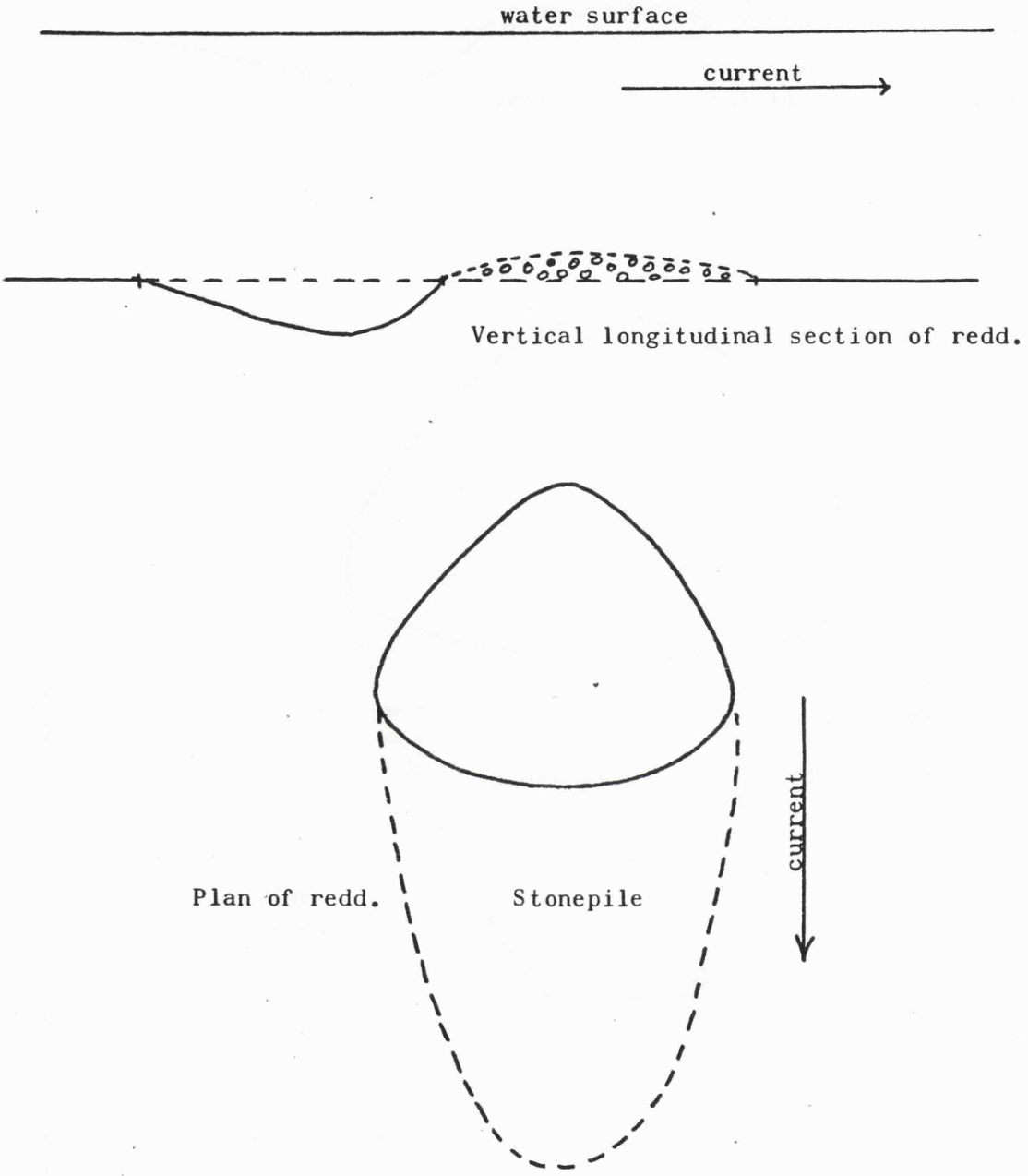


Figure 16 (continued). Diagram of the average L. planeri redd. (Scale = x 0.2)

(Facing p. 79)

TABLE II. Redd dimensions for marinus and planeri, with river depth and surface water velocity.

	Width (w)		Length (L)		Stonepile length (s)		Redd depth (d_4)		River depth (d_2)	Water velocity
Units	cm	f	cm	f	cm	f	cm	f	cm	cm
P. marinus										
Number of redds	11	11	11	11	11	11	11	11	8	33
Mean	148.18	1.98	102.36	1.36	104.82	1.40	15.10	0.2	30.75	77.57
$s/\sqrt{n}$	11.27	0.15	15.41	0.20	12.34	0.16	1.16	0.016	3.43	3.61
L. planeri										
Number of redds	18	18	19	19	16	16	18	18	18	43
Mean	26.53	2.10	22.18	1.75	23.31	1.84	4.22	0.33	17.19	35.93
$s/\sqrt{n}$	3.00	0.24	1.76	0.14	3.24	0.26	0.34	0.027	1.27	1.26
Significance of differences	$P<0.001$	n.s.d.	<0.001	n.s.d.	<0.001	n.s.d.	<0.001	n.s.d.	<0.001	<0.001

f = 'animal length units'. marinus taken as 75cm long and planeri as 12.65cm long. Hence the mean width of a marinus redd is given as 148.18cm and 1.98f (= 148.18/75).

(Facing p. 79)

B Redd measurements

The average redds for planeri and marinus are illustrated in Fig.16 which shows the plans of the redds and also a vertical longitudinal section (i.e. parallel to the current flow) through the deepest part of the redd.

The measurements are summarised in Table II for both animals, results being given in cm and also as functions of the mean lengths of the animals (f columns in the Table).

As might be expected, there is a very highly significant difference between dimensions for the two animals when the comparisons are between absolute units ($p < 0.001$). It would be surprising if there were not these differences because of the great size difference between the animals. However, if the redd dimensions are expressed as function of the average lengths of the animals, these differences disappear, suggesting that the patterns of the redds do not differ between the two species except in magnitude. All comparisons were made with Student's t-test for small samples.

A particularly interesting comparison is between redd volumes, given in Table III. These results suggest that the volume of the redd is related firstly to the number of animals using it, as might be expected; and secondly to the spawning activities and behaviour of the animals. If redd volume were directly related to the volume of the animals, one would expect column ii in the Table to show little difference. On the other hand, if redd volume depended upon the 'swept' volume of the redd because of the activity of the animals, it would be related to the cube (or near function) of the length of the animals. This cubic relationship is shown by column iii

	(i) Volume (cc)	(ii) Volume/* (cc)	(iii) Volume/ø (cc)
P. marinus			
Number of redds	11	11	11
Mean volume	62347	13.30	0.072
$s/\sqrt{n}$	17544	3.74	0.018
L. planeri			
Number of redds	18	18	18
Mean volume	910.1	28.26	0.080
$s/\sqrt{n}$	223.8	7.26	0.021
Significance of difference	$p<0.001$	$p<0.01$	n.s.d.

TABLE III. Redd volumes of marinus and planeri redds. Column ii gives the volume parameters divided by the average volume of the animal and the average number of animals in the redd (= *). Column iii gives the volume parameters when the redd volumes are divided by the cube of the length of the average animal times the average number of animals in the redd (= ø). The significances of the differences are indicated and discussed in the text.

(Facing p. 80)

of the Table, which reveals no significant difference between redd volumes. Such a relationship could have been hypothesised and it would be interesting to have measurements of fluviatilis redds for comparison since they are intermediate in length between marinus and planeri. These comparisons can only be fruitful if the numbers of animals in redds can be taken into account. P. marinus redds have been assumed to have 2.5 occupants on average, and the planeri redds measured contained an average of 5.6 animals. It should be emphasised that the statistical tests used can suggest differences but cannot place a probability on similarities, however, the change in probabilities is indicative that the behaviour as well as size is an important factor in determining redd volumes.

The volume of the redd must be closely related to the size of the stonepile downstream from the excavation. Only a part of a stonepile was collected from a marinus redd. The average weight (in air) of the stones was 490g, and the average density in air was 2.23. The effective density in water (i.e. allowing for the water displaced) would be about 1.23.

The average volume of a marinus redd is 62.31 and therefore the weight (in water) of stones excavated would be about 70kg making an allowance for the packing of the stones. The largest stone found in the stonepile was 2.35kg (in air) and it had a volume of 1050cc, and therefore an effective weight in water of 1.30kg. A particular stone may be moved more than once during the construction of a redd as its boundaries are gradually extended, so the work done is quite considerable.

L. planeri have been seen collectively moving a stone which

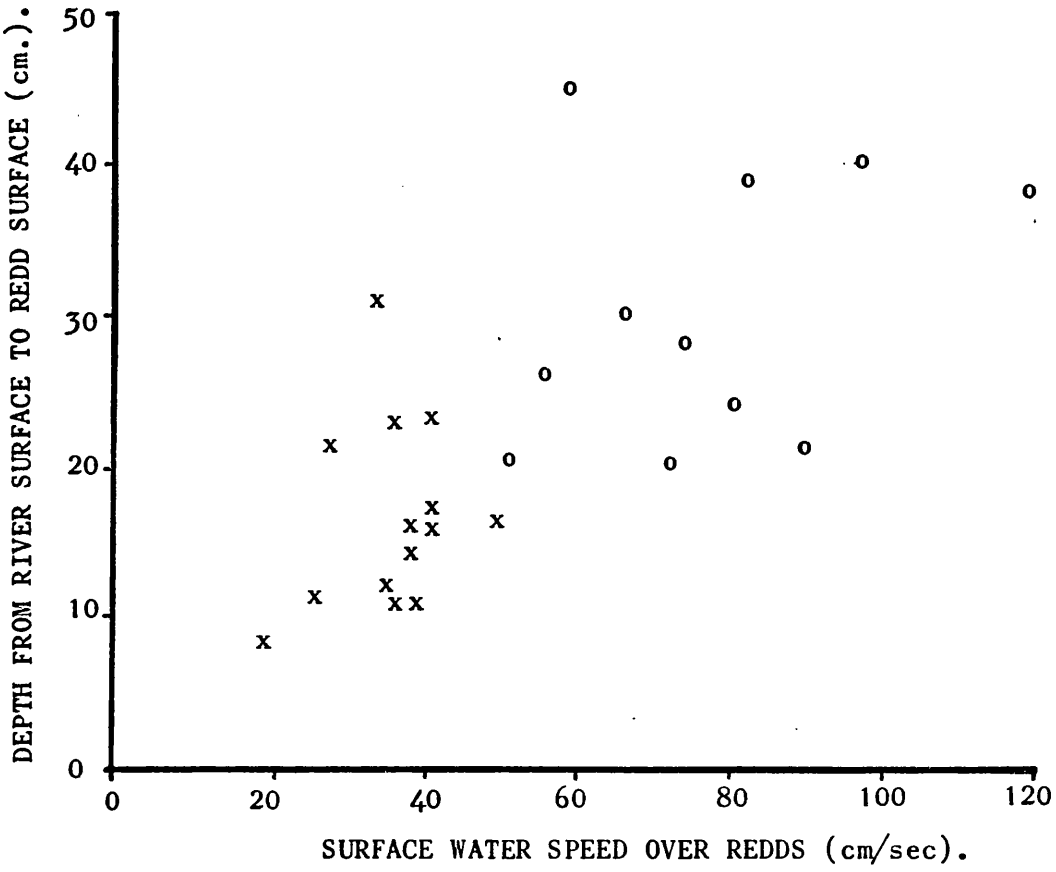


Fig. 17. Scatter diagram of marinus redds (o) and planeri redds (x) on water depth to redd surface and river surface water speed axes.

(Facing p. 81)

was too large to be moved by an individual but this has not been observed in marinus redds.

During the excavation of the average planeri redd, about 1kg of stones, gravel and sand will have been removed. In planeri stonepiles the mean weight of stone (in water) removed was 1.6g and the heaviest (in water) found was 6.3g. In one of the redds sampled, four stones remained on the floor and these weighed in water, 10.7, 9.9, 9.7 and 9.5g respectively. These stones are interesting from two points of view. Firstly, since they were the only stones remaining in a well populated redd (10 animals taken from the redd) they were probably beyond the 'removal capacity' of planeri even with 'collaborative' efforts of more than one animal. Secondly, the stones represented a significant discontinuity when compared with the other stones in the pile ($p < 0.001$). It implies that the size of the stones of the substratum is critical in the suitability of the site for a redd. The overwhelming majority of the stones must be of such a size that they can be removed (either by the sucker or by tail-fanning - see Spawning Behaviour results) and suitable regions in a river are likely to be few. Most redds had sandy floors with no remaining stones.

This implies that the dimensions of the stones on the river bed could effectively isolate spawning animals of different lengths if they have to excavate the redds for themselves, although a smaller animal could use a redd already prepared by a larger animal. The implications of these points are more fully explored in the discussion.

Fig. 17 is a scatter diagram showing the distribution of planeri

and marinus redds on coordinates of water depth and water speed (surface). The ranges for the two animals overlap in depths but not quite in surface water speeds. These 'preferred' conditions are probably connected with the stone-sorting action of the river as well as with the stone moving abilities and physical dimensions of the animals.

It would be interesting to add a scatter diagram for fluviatilis redds but since their spawning and redd building has not been adequately observed, this is not possible.

There is a close correlation between surface water speed and the depth of the river over the redds ($p < 0.001$ of it happening by chance). However, the correlation between surface water velocity and the depth from the floor of the redd to the surface of the river is even higher ($p \ll 0.001$). This reinforces the suggestion that the characteristics of the stones are of primary importance in the selection of a site. Surface water velocity is of secondary importance since unsuitable speeds within the redds can be compensated for to a limited extent by the excavation of a deeper or shallower redd as is appropriate.

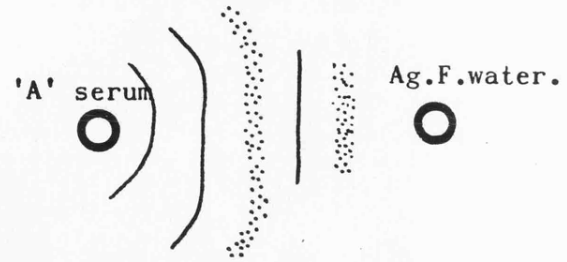


Figure 18. For explanation of this and similar double gel diffusion Figures, please see the text.

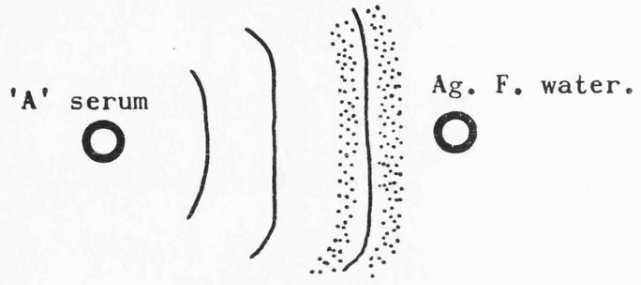


Figure 19.

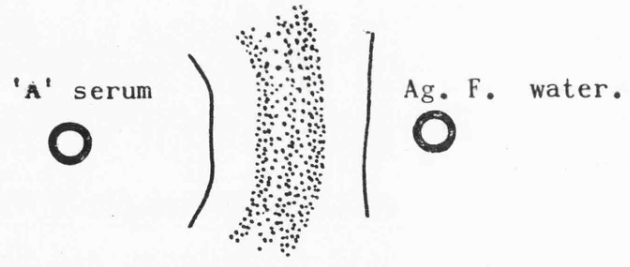


Figure 20.

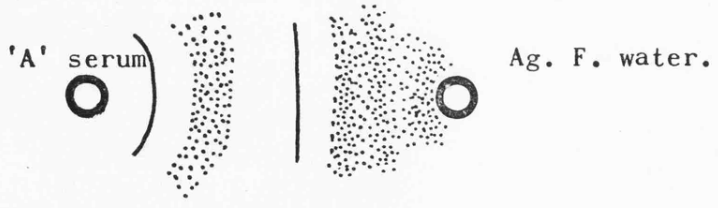


Figure 21.

On these and subsequent Figures ○ = well.

(Facing p. 83)

C Gel diffusion

Plate double-diffusion in gel was used during preliminary investigations of the relationships among various lamprey mucuses.

Anti-fluviatilis serum 'A' was tested against freeze dried fluviatilis mucus collected with water and reconstituted in borate buffer to give a 0.06% w/v concentration (Campbell, Garvey, Cremer and Sussdorf, 1964). The first tests showed that the mucus is a multiple antigen and five different precipitin bands were distinguished (Fig. 18).

As the technique was mastered, more precipitin lines were distinguished but it was soon apparent that serum 'A' did not give consistent results. Six rabbits contributed to this serum and as rabbits were later found to respond with different titres to mucus, it is likely that these pooled antisera had different antibody levels to the various antigen systems at different times.

As the differences were due to the absence of some lines at one or other end of the pattern, rather than to completely different patterns (Figs. 19, 20 & 21) it was possible to build a composite picture of the precipitin pattern (Fig. 22).

Ethanol-collected mucus, which later became the standard fluviatilis antigen for immunisations and immunoelectrophoresis, was compared with water-collected mucus against anti-fluviatilis serum 'A'. Two lines present in water-collected mucus were not found in ethanol-collected mucus (lines 4 & 6, Fig. 22), which itself had one line not present in water collected mucus (line 2b in Fig. 22), and not related to its nearest neighbours (Fig. 23). When dealing with the diffuse lines that were

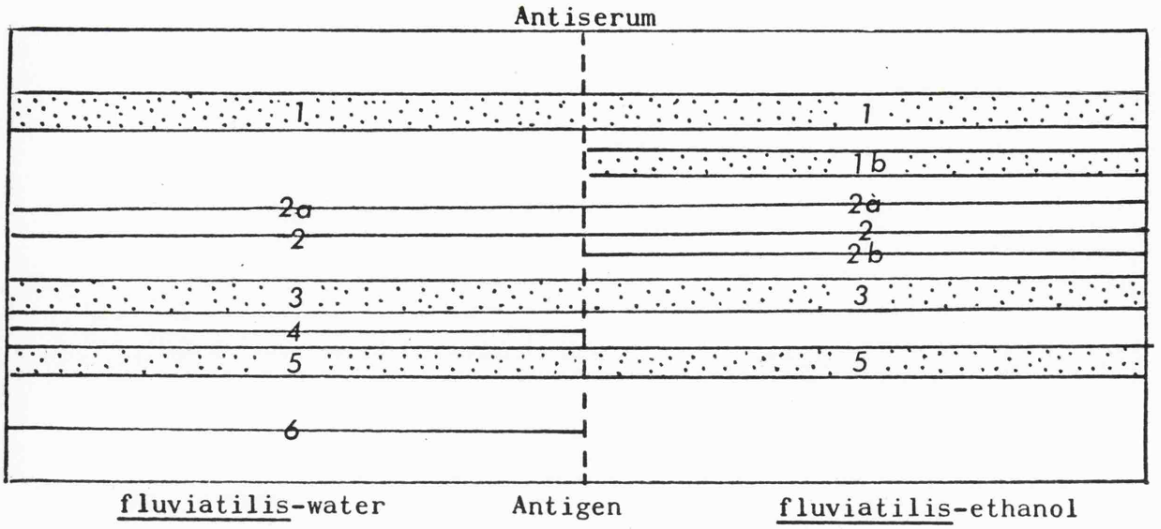


Figure 22. To show the composite patterns found on gel diffusion, contrasting the precipitin lines with fluviatilis antigens collected with water and ethanol.

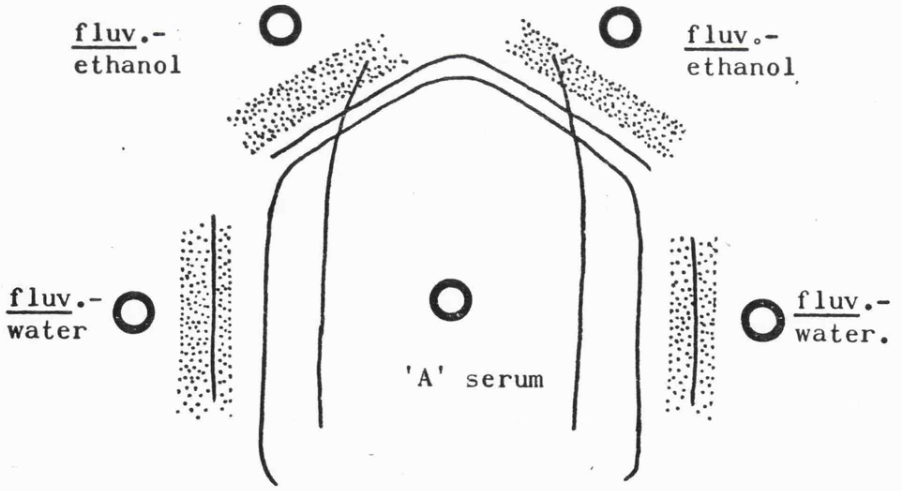


Figure 23. To show typical double gel diffusion patterns against 'A' serum, of water- and ethanol-collected mucus of fluviatilis.

(Facing p. 84)

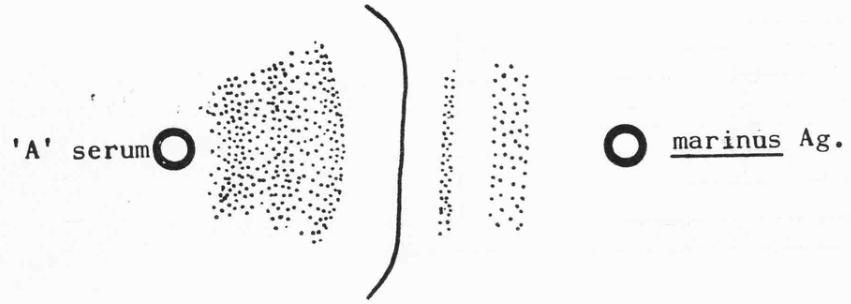


Figure 24.

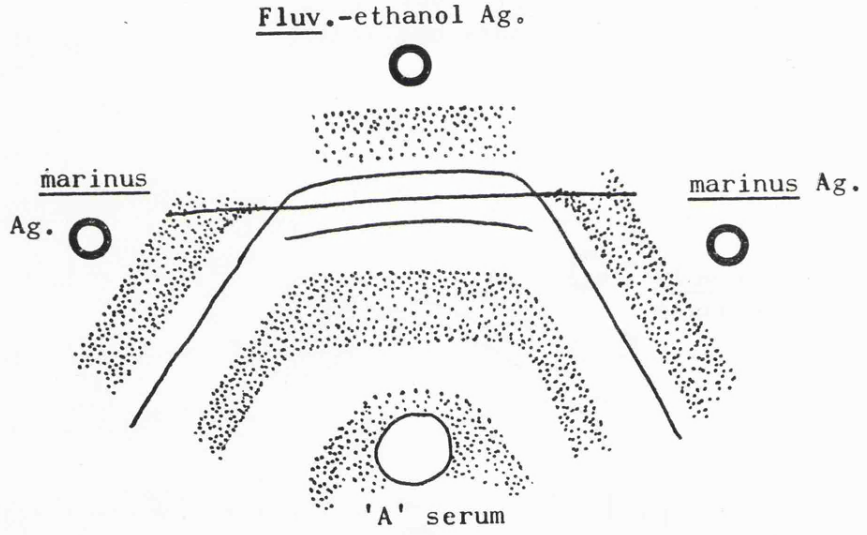


Figure 25.

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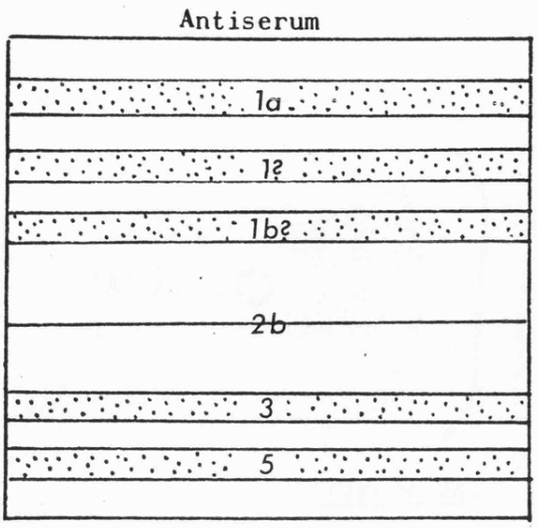
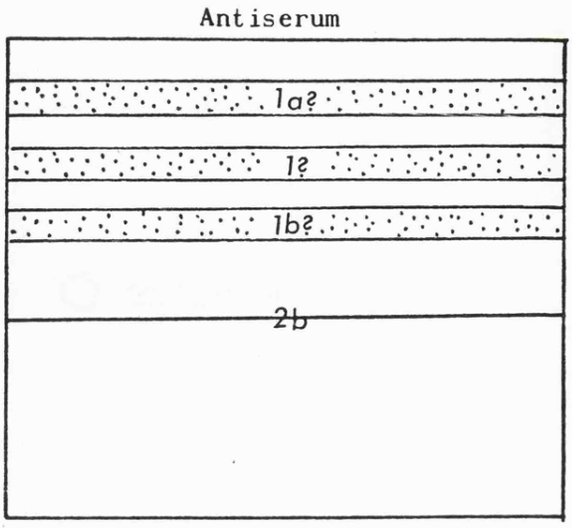


Composite pattern for marinus mucus.

Figure 26.



Composite pattern for planeri mucus.

Figure 27.

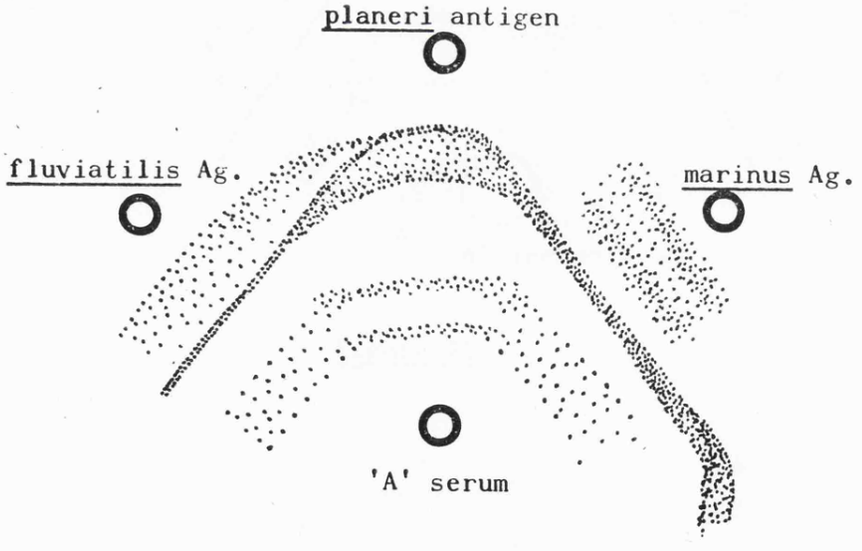


Figure 28. To show the precipitin pattern obtained with 'A' serum against antigens from the three lampreys.

(Facing p. 84)

common in these gel diffusion patterns, it is not always possible to see correspondence, especially if lines are on the outside of the 'spectrum', neither is it always possible to distinguish between single or double lines; e.g. lines 3, 4 & 5 (Fig. 22) often appeared as a single, broad and fairly dense line.

Freeze dried marinus mucus (reconstituted to 0.06% w/v in buffer) when tested against anti-fluviatilis 'A' serum showed a pattern of six lines (Fig. 24), of which three probably corresponded with those in fluviatilis, two did not (Fig. 25) and the sixth line (line 1a in Fig. 26) had not been found in fluviatilis, although antibody to it or a closely related antigen, must have been present in 'A' serum.

Raw planeri mucus gave four lines against anti-fluviatilis 'A' serum (Fig. 27) but it is likely that the concentration of antigen used was too low to give optimum results. At least one of these lines corresponded to one in marinus and fluviatilis but the pattern was difficult to interpret (Fig. 28).

The initial results suggested therefore, that the mucus of the three lampreys is very similar in that several of the antigens are common, as could be expected, but that some of the antigens might be species-specific, although closely related.

Because of the difficulty of getting reproducible results and because comparison of the patterns is difficult with so many diffuse bands, subsequent analyses used the more sensitive technique of immunoelectrophoresis.

ANTIGENS			Anti-fluv. (adult mucus)							Anti-mar. (adult mucus)							Anti-plan. (amm. muc.)		
		ANTISERA	'A'	F17	F18	F19	F20	F21	F22	M6	M7	M8	M11	M13	M14	M23	P2	P3	P4
	fluviatilis	adult mucus	X	X	X	X	X	X	X	X	X	X	X	X	X		X	X	X
		ammocoete mucus															X		
		adult skin																	
		ammocoete skin									X						X		
	marinus	adult mucus	X	X	X	X	X	X	X	X	X	X	X	X			X	X	X
		ammocoete mucus									X						X		
		adult skin								X									
		ammocoete skin								X							X		
	planeri	adult mucus									X								
		ammocoete mucus	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		adult skin									X						X		
		ammocoete skin									X						X		
	Sundry species	Anguilla mucus	X								X						X		
		Gadus sp. mucus															X		
		Salmo smolt mucus															X		
		Platessa mucus	X																

TABLE IV. Antigen/antisera crosses tested. Rabbit M23 died before useful quantities of serum could be collected.

(Facing p. 85)

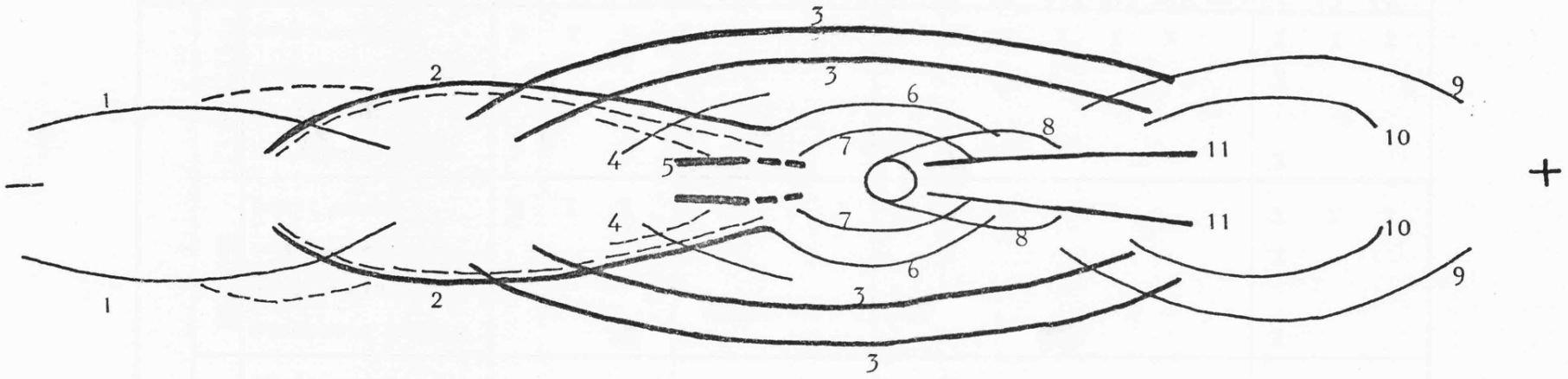


Fig. 29. A composite pattern of precipitin lines obtained in the immunoelectrophoresis experiments. The dashed lines occur infrequently. The origin and polarity are indicated. Line 3 is shown twice since it is variable in position between these two extremes.

(Facing p. 85)

D Immunoelectrophoresis

The antigen-antiserum combinations ('crosses') tested are given in Table IV. The precipitin patterns formed by any given antigen/antiserum combination after immunoelectrophoresis sometimes varied, occasionally considerably. The probable causes of this variation are considered below.

It is possible to relate the variations, and the typical patterns formed by particular antigen/antiserum reactions to a composite pattern (Fig. 29). This composite was formed by comparing patterns and plotting all the frequently occurring precipitin lines. Lines were identified by their positions relative to one another and to the origin, and by their usually characteristic appearance, i.e. some lines were always sharply defined while others were blurred; some lines were arcuate and some always straight. Precipitin lines represented in Fig. 29 by dotted lines are those that appeared only occasionally and inconsistently in any given antigen/antiserum cross.

1 Variability of patterns

a) Sera

The variability of patterns obtained in each antigen/antiserum cross was the result of differences in the reagents and the technique.

Antisera were more variable than antigens. The original 'A' antifluviatilis serum was obtained by pooling the blood from six rabbits, bled at weekly intervals. Each batch of 'A' serum gave slightly different results from the other batches,

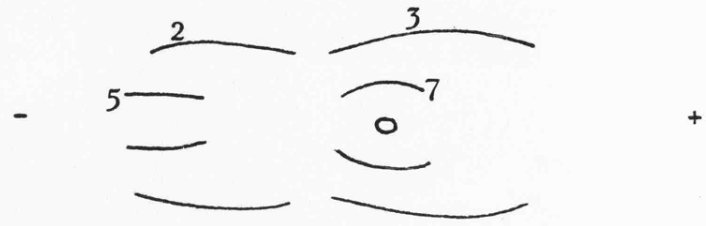


Fig. 30. FAm/F'A'.

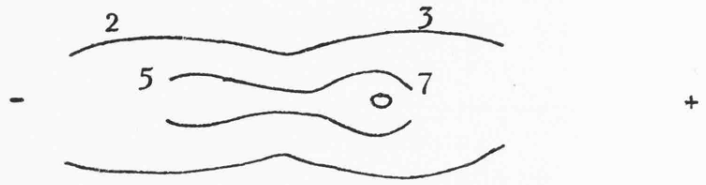


Fig. 31. FAm/F'A'.

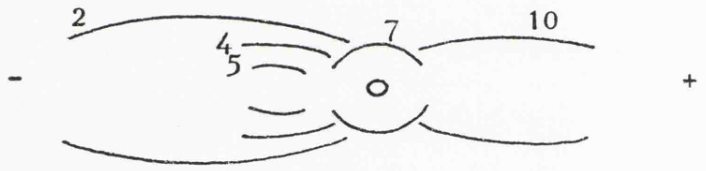


Fig. 32. MAm/M7.

Fig. 33. MAm/M8.

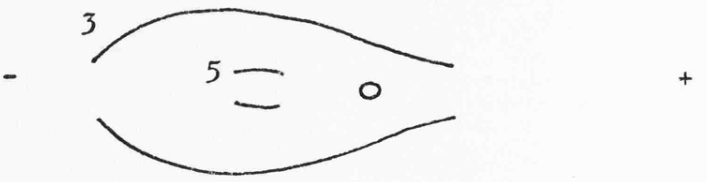


Fig. 34. FAm/F17.

(Facing p. 86)

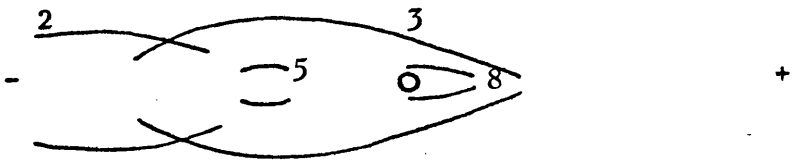


Fig. 35. FAm/F19.

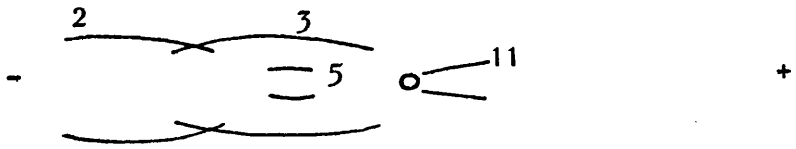


Fig. 36. Pam/F19.

Fig. 37. Pam/F22.

(Facing p. 86)

e.g. fluviatilis antigen against anti-fluviatilis 'A' (Fig 30) was an early batch of 'A' serum, whereas Fig. 31 was slightly different in pattern as a result of the use of a later batch of 'A' serum agains the same antigen.

For the identification of crosses in the figures and text in this section of the results, the following abbreviations will be used:-

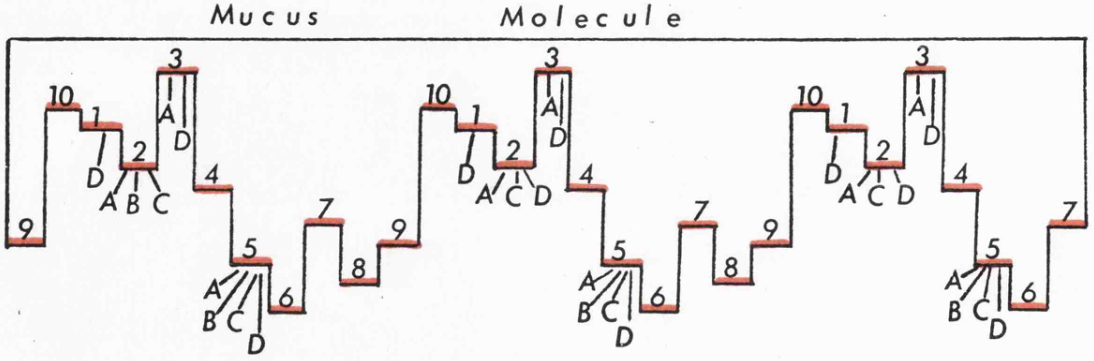
in antigen identification
F = fluviatilis
P = planeri
M = marinus
a = ammocoete
A = adult
m = mucus
sk = skin extract

in antiserum identification
/F = antifluviatilis serum
/P = anti-planeri serum
/M = anti-marinus serum
the number identifies the rabbit
'A' relates only to the pooled sera described above.

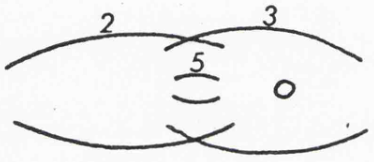
For example, Fam/M7 = fluviatilis ammocoete mucus antigen against anti-marinus serum from rabbit 7; PAsk/P2 = planeri adult skin extract as antigen against anti-planeri serum from rabbit 2.

The sera from individual rabbits were subsequently used separately and great variation in the precipitin patterns was evident; e.g. Fig. 32 (MAm/M7); Fig. 33 (MAm/M8); Fig.34 (FAm/F17); Fig. 35 (FAm/F19); Fig. 36 (Pam/F19) and Fig. 37 (Pam/F22).

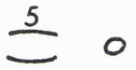
One of the causes of this variability was a function of the rabbits' sensitivity to the antigen generally. When given the



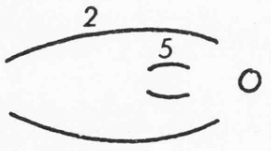
a)



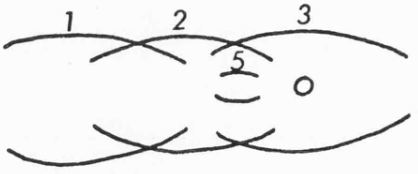
Rabbit A (Fig. 38b).



Rabbit B (Fig 38c)



Rabbit C (Fig. 38d)



Rabbit D (Fig. 38e)

Figure 38. A suggested explanation for the differing reactions among Rabbits A to D to a mucus molecule with multiple antigenic sites.

(Facing p. 87)

same immunisation treatments, some rabbits produced high titre sera, while others produced sera in which antibody was barely detectable. In this context it was noted that the New Zealand White rabbits ('A', M6, P2, P3 & P4) appeared to be more sensitive to this antigen than did the Californians, of which only two, M7 and M8, both from the same litter, produced good antisera. The others, all of the Hylyne strain, responded rather poorly to immunisation (F17, F18, F19, F20, F21, F22, M11, M13, M14 and M24).

However, differences in titre alone could not account for all of the variation found in these patterns. It is possible that mucus is a multi-site antigen and that some rabbits react to some sites more readily than to others. In Fig. 38a, mucus is represented as a large molecule with ten antigenic sites shown in red. When immunised the rabbits will tend to produce antibody most readily to certain sites only, say 2,3 and 5 (rabbit A in Fig. 38b). Some rabbits may produce antibody to one site only, e.g. rabbit B to site 5 (Fig. 38c); or to two, e.g. rabbit C to sites 2 & 5 (Fig. 38d). Although antibody to line 3 may be produced in rabbit C, the titre is too low to give a visible precipitate. Another rabbit (D in Fig. 38e) while showing the usual response to 2,3 & 5 may also be especially sensitive to, say, site 1 and will give the pattern shown.

In any given group of antisera, therefore, there were differences in the precipitin pattern produced with any one antigen. It proved possible however, to use each antiserum separately with different antigens, and produce patterns that could be directly compared.

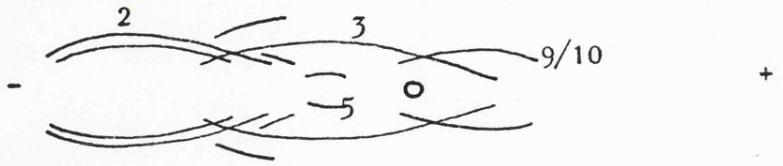


Fig. 39. FAm/P2.

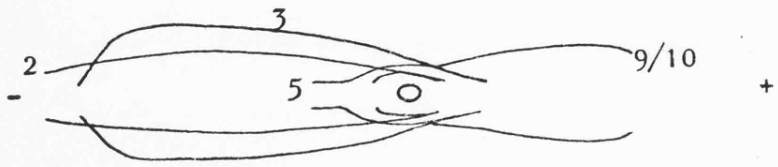


Fig. 40. FAm/P2 - High voltage.

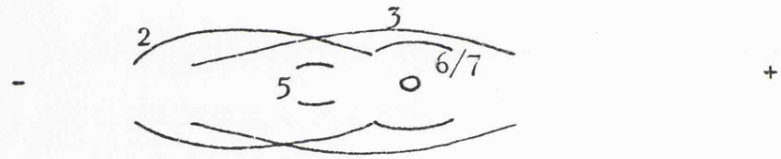


Fig. 41. Fam/P2.

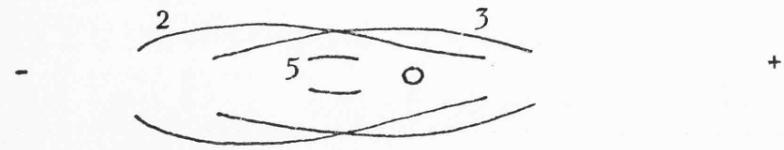


Fig. 42. FAm/P2.

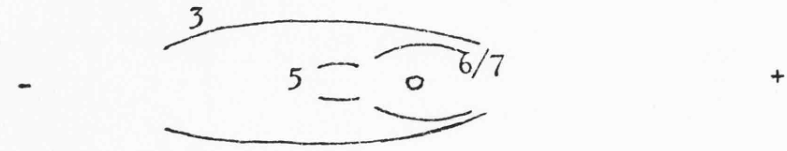


Fig. 43. Fam/M7.

(Facing p. 88)

Antigen	antiserum	precipitin lines 1	2	3	4	5	6	7	8	9	10	11
fluviatilis												
adult mucus	fluviatilis		X	X		X		X	X			X
adult mucus	marinus		X	X		X			X			
adult mucus	planeri		X	X		X				-X-		X
ammocoete mucus	marinus			X		X	-X-					
ammocoete mucus	planeri		X	X		X	-X-					
ammocoete skin	planeri		X	X		X	X					
marinus												
adult mucus	marinus	X	X		X	X		X		X	X	
adult mucus	planeri	X	X			X		X				
adult mucus	fluviatilis		X			X		X				
ammocoete mucus	marinus	X	X			X		X				
adult skin	planeri		X			X	X	X		-X-		
ammocoete skin	planeri		X	X		X	X	X		X	X	
planeri												
ammocoete mucus	planeri		X	X	X	X	X			X		X
ammocoete mucus	fluviatilis		X	X		X						X
ammocoete mucus	marinus		X	X		X						
adult skin	planeri		X	X		X	X			X		
ammocoete skin	planeri		X	X		X	X	X		X		X
adult skin	marinus		X	X		X	X	X				
ammocoete skin	marinus		X			X	X	X				

TABLE V. The occurrence of precipitin lines in the tested crosses. X indicates a precipitin line and -X- indicates that the identity of that particular line is in doubt but it will be one or other of the lines indicated.

(Facing p. 88)

b) Antigens

Variability in the antigen seemed insignificant, despite the use of raw mucus from adult planeri and ammocoetes, and the use of skin extracts, all of unknown protein content. Presumably the appearance of precipitin only at the equivalence point of the antigen/antibody reaction largely cancelled out this source of variation. The antigenicity of mucus solutions stored in the deep freeze did not decline.

c) Technique

The technique used was as standardised as possible but initial and final voltages varied from run to run, possibly due to differences in ambient temperature and thickness of gel. Where initial voltages were high (400v) the pattern was distorted though seldom unrecognisably so, e.g. FAm/P2 of Fig. 39 compared with FAm/P2 (high voltage) in Fig. 40. Such high voltage runs were infrequent.

2 Composite pattern

Despite these sources of variation, it was possible to refer all of the patterns obtained to the composite shown in Fig. 29. Table V shows the occurrence of the precipitin lines in the various antigen/antibody crosses tested. Line 5 was ubiquitous and consistent, both in its position near the midline and less than one third of the distance from the origin to the negative pole; and in its character - thick, rather blurred and usually linear rather than arcuate. It was usually the first line to appear after immunisation and in weak antisera it was often the only line.

Almost equally common was line 2. Its position was rather more variable than that of line 5 although it was consistent relative to other precipitin lines. Unlike line 5, it was a thin, well defined arc. Its absence in Fam/M7 was probably because of the weakness of the antigen, as raw mucus from single ammocoetes was used and only small quantities of dilute mucus could be collected.

Lines 2 and 5 were useful as 'markers' in identifying other lines, with line 2 being more useful than line 5 in this respect. Neither, of course, was of use in distinguishing among lampreys as they were not even genus specific.

a) Ammocoete and adult comparisons

Adult mucus was used as an immunising agent for anti-*fluviatilis* and anti-*marinus* sera, while ammocoete mucus was used in raising anti-*planeri* sera. It was therefore necessary to compare adult and ammocoete patterns lest this was responsible for any difference in the patterns given by the three lampreys. Skin extracts were used in this comparison as well as mucus, as the latter was available in such small quantities from ammocoetes. /M7 and /P2 were used as both had high titres and gave many lines when tested against their homologous antigen.

i) *fluviatilis*

fluviatilis ammocoete and adult mucus tested against /P2 both showed lines 2, 3 and 5. (Figs. 41 & 42). Fam/P2 also showed an 'eyebrow' line, probably number 7, as did both adult and ammocoete mucus when tested against /M7 (see Figs 43 & 44). In the adult mucus, one of these eyebrows had spread somewhat towards the positive pole and was therefore line 8. Fam/M7

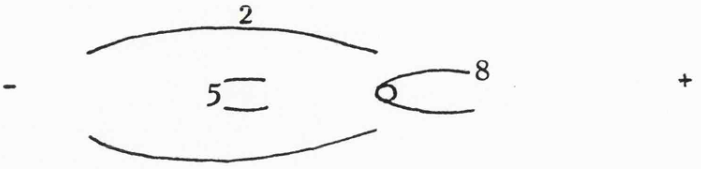


Fig. 44. FAm/M7.

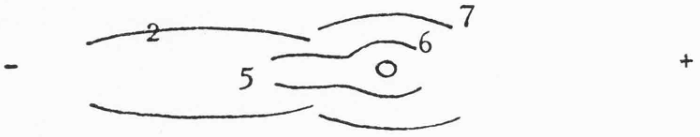


Fig. 45. Pask/M7

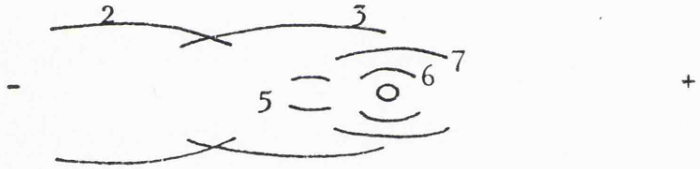


Fig. 46. PAsk/M7.

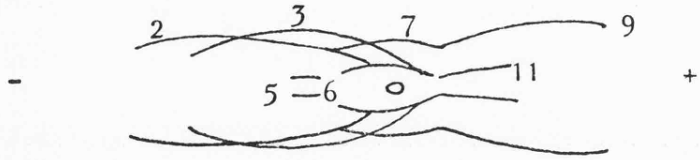


Fig. 47. Pask/P2.

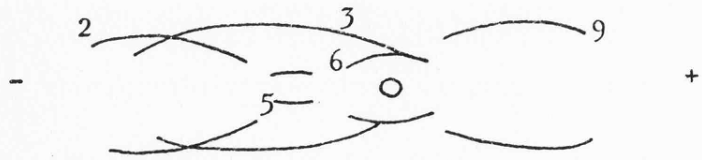


Fig. 48. PAsk/P2.

(Facing p. 90)

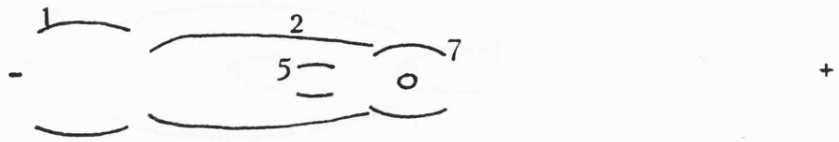


Fig. 49. Mam/M7.

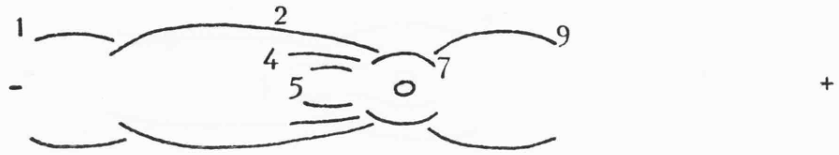


Fig. 50. MAm/M7.

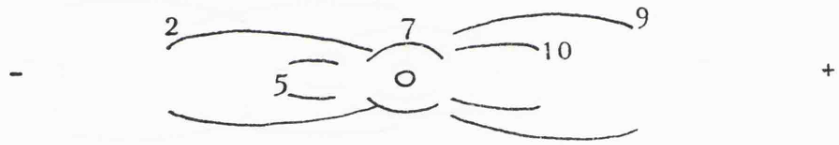


Fig. 51. MAm/M7.

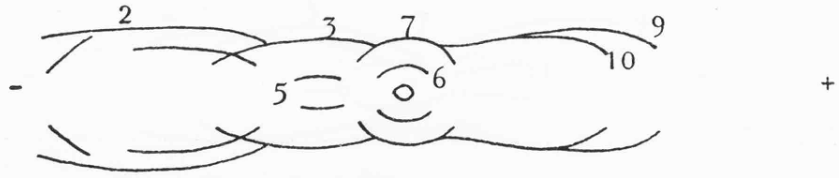


Fig. 52. Ma(piece of skin)/P2.

Fig. 53. MAsk/P2.

(Facing p. 90)

did not show line 2 but, as suggested above, this is probably because the test antigen was so weak. The total precipitin lines showed by Fam, and also by Fask, was 2, 3, 5 and 7 which correspond to those shown by adult mucus, which additionally has line 8.

ii) planeri

planeri adults were compared with ammocoetes by their skin extracts, as mucus was not easily collected from the adults; they were available for a short time only, and as they were in spawning condition when caught, contamination by milt or peritoneal fluid was unavoidable. Pask/M7 gave lines 2, 5, 6 and 7 as did the adult skin extract (Figs. 45 & 46). Ammocoete skin extract gave lines 2, 3, 5, 6, 7, 9 & 10 against/P2 (Fig. 47) but adult skin did not show 7 or 11 (Fig. 48). The total lines shown by planeri ammocoete skin extract was 2, 3, 5, 6, 7, 9 and 11 and by the adult skin extract, 2, 3, 5, 6, 7, and 9. Line 11 was missing in the adult, but it was at best a weak line and it was also missing in several ammocoete skin extract crosses against /P2.

iii) marinus

marinus ammocoete mucus against /M7 showed only lines 1, 2, 5 and 7, while the adult mucus gave these and also lines 9 and 10 and a possible 4 (Figs. 49, 50 and 51). As in fluviatilis ammocoetes, the mucus antigen was probably rather weak, especially as the Mask/P2 cross gave lines 2, 3, 5, 6, 7, 9 and 10 while

Antigen	Anti-serum	Precipitin lines 1	2	3	4	5	6	7	8	9	10	11
fluviatilis												
ammocoete mucus	P2		X	X		X	-X-					
adult mucus	P2		X	X		X			-X-			
adult mucus	F'A'		X	X		X	-X-					
ammocoete mucus	M7			X		X	-X-					
adult mucus	M7		X	X		X			X			
ammocoete skin	P2		X	X		X	-X-					
ammocoete skin + mucus totals	total		X	X		X	-X-					
adult mucus totals	total		X	X		X	-X-		X			
planeri												
ammocoete skin	P2		X	X		X	X	X		X		X
adult skin	P2		X	X		X	X			X		
ammocoete skin	M7		X			X	X	X				
adult skin	M7		X	X		X	X	X				
ammocoete skin + mucus totals	total		X	X		X	X	X		X		X
adult skin totals	total		X	X		X	X	X		X		
marinus												
ammocoete mucus	M7	X	X			X		X				
adult mucus	M7	X	X		(X)	X		X		X	X	
ammocoete skin	P2		X	X		X	X	X		X	X	
adult skin	P2		X			X	X	X		-X-		
ammocoete skin + mucus totals	total	X	X	X		X	X	X		X	X	
adult skin + mucus totals	total	X	X		(X)	X	X	X		X	X	

Table VI. The occurrence of precipitin lines against particular antisera and the total lines for groups of antigens against the listed antisera. X indicates a precipitin line and -X- indicates that the identity of that particular line is in doubt but that it will be one or other of the lines indicated. (X) was not consistently found.

(Facing p 91)

the MAsk/P2 cross gave only 2, 5, 6, 7 and 9 or 10 (Figs. 52 & 53). Total lines for ammocoete mucus and skin were therefore, 1, 2, 3, 5, 6, 7, 9 & 10 and for adult mucus and skin were 1, 2, 5, 6, 7, 9 & 10. Line 3 was not found in adult mucus when tested against other antisera and it seems likely that this is a true difference between the larval and adult mucuses. It is perhaps surprising that more differences were not found between adult and larval mucuses (comparable with those of the haemoglobins) of the anadromous species, as the adults of both fluviatilis and marinus have a marine phase.

The results of the adult/ammocoete comparisons are given in Table VI.

b) Sex differences

Mucus from adult marinus and fluviatilis of both sexes were compared within the species for sex differences in the mucus, but none was found.

3. Specific patterns

a) marinus

The patterns given by marinus adult mucus with any serum differed from those given by adult fluviatilis and ammocoete planeri mucuses in several distinct ways.

This difference was most striking when using anti-fluviatilis 'A' serum. Apart from line 5, no precipitin appeared on the negative side of the origin in M/F'A' crosses, while in F/F'A' crosses, all lines were on the negative side (compare Fig. 54 with Figs 30 & 31) and in P/F'A' all but one were on the negative

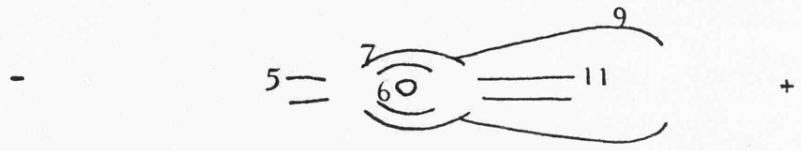


Fig. 54. MAm/F'A'.

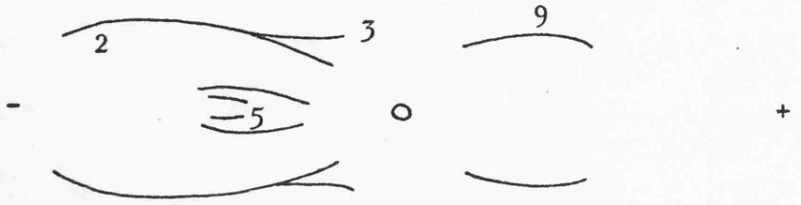


Fig. 55. Pam/F'A'.

Fig. 56. MAm/P2.

Fig. 57. MAm/F19.

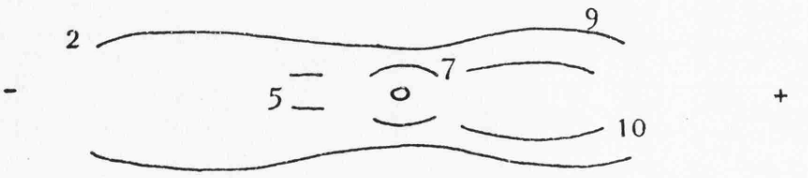


Fig. 58. MAm/M6.

(Facing p. 92)

side (Fig. 55). The eyebrow line, 6 or 7, was always present in marinus while only occasionally so in fluviatilis and not at all in planeri. The eyebrow present in fluviatilis was not that found in marinus, as mixed marinus and fluviatilis antigens gave a double line at this site (see Fig. 71).

Although these distinctions were not exactly duplicated using later sera, the patterns given by /F'A' are a reflection of a real quantitative and perhaps qualitative difference between the mucus antigens of marinus and those of fluviatilis and planeri.

Adult marinus mucus run against later /F, /P and /M showed four features distinguishing it from fluviatilis or planeri mucus. Line 1 was present when marinus mucus was run against /P (Fig. 56); whereas fluviatilis and planeri did not give this line with any serum. Secondly, line 3 was never present in marinus, but always in fluviatilis and planeri. An eyebrow line, identified as line 7, was always present in marinus crosses (Fig. 57); such a line was only found in one fluviatilis cross (/F'A') and not at all in planeri mucus crosses. As mentioned above, the eyebrow line in fluviatilis was not the same as that found in marinus. Lastly, M/M crosses showed lines 9 and 10 (Fig. 58) whilst fluviatilis and planeri showed only one line, against /P, in this anodic position.

With the other marinus antigens - ammocoete mucus and skin extracts - and adult skin, the position was more complicated. Mam/M showed line 1, but was not tested against /P; however, fluviatilis and planeri skin extracts and mucuses did not give this line. Mam did not give lines 9 or 10, perhaps because its was a weak antigen. Apart from this difference, ammocoete

mucus showed the same differences from planeri and fluviatilis as the adult.

Skin extracts of adult and ammocoete marinus differed from adult mucus in not showing line 1 against /P. marinus ammocoete skin extract against /P showed line 3, but in other respects, skin extracts could be distinguished from those of planeri and fluviatilis by the same criteria as adult mucus.

It is likely that these distinctions are the result of quantitative differences in the mucus antigens, rather than their presence or absence in any given mucus. For instance, as stated above, line 3 is almost always absent in marinus antigen crosses, the exception being the ask/P2 cross. Skin extracts were normally more powerful than mucus in that the precipitin lines formed were denser, better defined and usually more numerous than in the corresponding mucus cross. The antigen responsible for line 3 is, in fact, present in marinus adult mucus, as crosses of planeri and fluviatilis antigens against /M showed this line. Clearly, the quantities of antigen needed to provoke an antibody response are smaller than those needed to form a visible precipitate. Again, line 10 is not present in any pattern formed by planeri mucus, although antibody to it is nevertheless present in anti-planeri serum, as marinus mucus and skin extracts against anti-planeri serum show it.

Whatever the reasons for the differences shown in these patterns, they are consistent enough to allow unequivocal identification of marinus antigens when tested against any anti-serum.

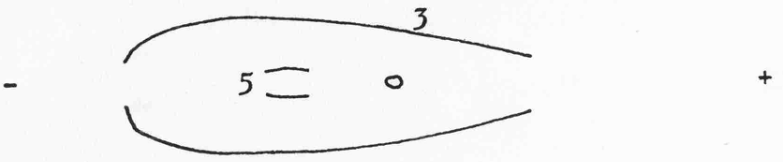


Fig. 59. FAm/F17.

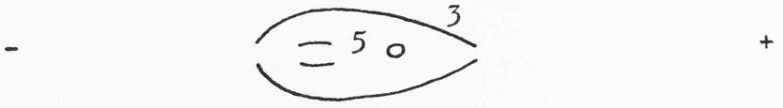


Fig. 60. Pam/F17.

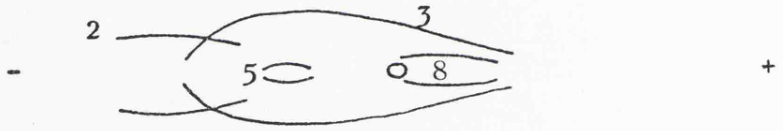


Fig. 61. FAm/F19.

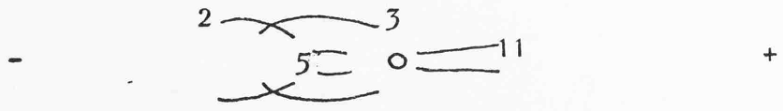


Fig. 62. Pam/F19.

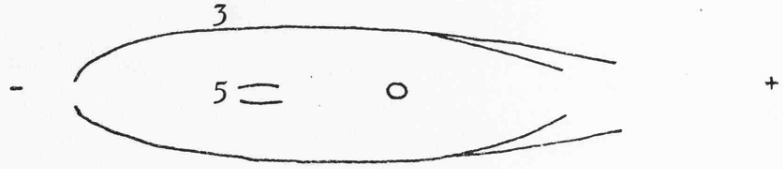


Fig. 63. FAm/F21.

(Facing p. 94.)

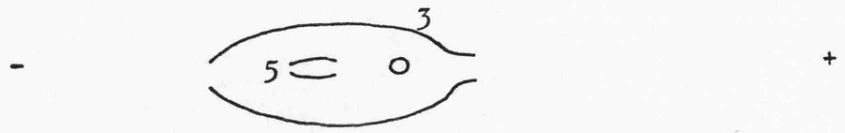


Fig. 64. Pam/F21.

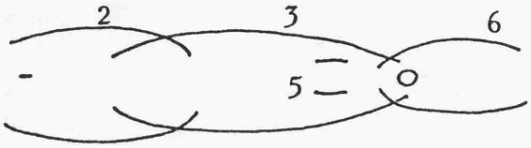


Fig. 65. Pam/P2.

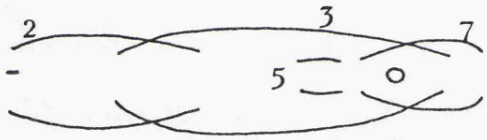


Fig. 66. FAm/P2.

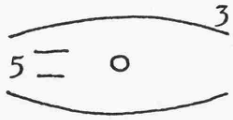


Fig. 67. Pam/F21

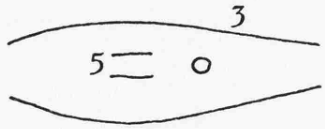


Fig. 68. Fam/F21.

(Facing p. 94)

b) fluviatilis/planeri

In contrast with the ease of identification of marinus, it was not possible to consistently and unequivocally distinguish between planeri and fluviatilis with any antiserum except/M7. Differences between planeri and fluviatilis antigens were shown by various sera, e.g. the series/F17 to /F22 (Figs 59 to 64). In this case there is a clear difference in the nature of line 3 in the two crosses of all of this series of antisera. However this and other differences of the same nature were very elusive on detailed investigation. It appears from the figures that line 3 here is in fact two different lines, but this is not supported by other crosses with the sera or the antigens. Line 3 is somewhat variable in position and type and it is quite possible that there are several antigens responsible for this line. On close comparison, in fact, it would seem that there are as many line 3's as there have been electrophoretic patterns produced! Line 3 in the P/F crosses could be a truncated version of that in the F/F crosses; and, as stated above, planeri mucus was used raw because of its scarcity and it is possible that the difference in these patterns is a reflection of different antigen strengths.

For these reasons, although this series of sera does produce different patters for fluviatilis and planeri, it was felt that a close examination of /M7 patterns would be more fruitful.

Figures 65 to 68 illustrate the general inability of antisera to distinguish between planeri and fluviatilis antigens.

/M7 did distinguish between the two in that an eyebrow line,

Fig. 69. Pam/M7.

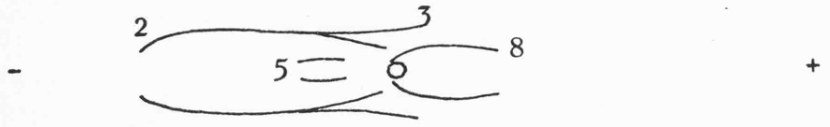


Fig. 70. FAm/M7.

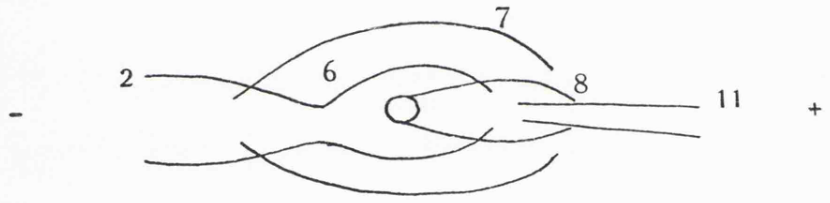


Fig. 71. Eyebrow detail (on larger scale).

(Facing p. 95.)

number 8, consistently occurred in the fluviatilis cross but never in the planeri cross (Figs. 69 and 70). It was not a typical eyebrow, i.e. 6 or 7, having run slightly towards the positive pole (Fig. 71), a movement not found in other patterns. F/F crosses did show line 8, although other fluviatilis crosses showed no eyebrows. Skin extract planeri crosses usually showed an eyebrow, two in some cases (Fig. 46).

c) Teleosts

Mucus of Gadus sp (probably G. calliaris), Pleuronectes platessa, Anguilla anguilla and Salmo salar were tested against /F'A' and /P2, but in no case could precipitin be detected.

4. Summary of immunoelectrophoresis results

The results reported above largely reflect what is known of the relationships of lampreys. The teleosts tested gave no result with anti-lamprey sera. This was the expected result in view of the accepted theory of the origins of the Cyclostomes and the long period of their divergence from Teleost stocks.

Petromyzon marinus is placed in a different genus from Lampetra planeri and L. fluviatilis and this separate taxonomic status is supported by the pattern shown by the mucus. The lampreys appear to be a relatively homogeneous group however, and it is not surprising that so many of the mucus antigens are held in common.

The differences between marinus and the Lampetras are significant however, and when compared with those between planeri and fluviatilis agree well with the separate generic status of marinus.

Fluviatilis and planeri are undoubtedly very closely related, but it is difficult to gauge how closely without a comparison of a similar degree of relationship. Attempts were made to test the mucus of a probable 'praecox' form of fluviatilis, but insufficient quantities of mucus could be collected.

The origins of planeri and the taxonomic status of planeri and fluviatilis will be considered in greater detail in the discussion.

E. Antibody preparation and fluorescent labelling

During gamma globulin precipitation, some antibody activity was lost, as is usual. Fluorescent labelling of the gamma globulin also reduced the quantity of antibody present. Although the labelled anti-*marinus* antibody produced did show some activity against *marinus* antigens, it was not of sufficient titre to show specific differences between *planeri* and *fluviatilis* antigens.

This disappointing result with fluorescent labelling suggested the need for practical expertise in these isolation and labelling techniques before further scarce reagents were used.

Successful fluorescent labelling has been achieved using the cyanogen bromide technique, of specific antibody preparation with human IgG and anti-human IgG sera. This was part of another research programme, the results of which will be reported elsewhere.

Now that practical expertise has been acquired, it is hoped that there will be an opportunity for applying the techniques to lamprey antigens and antisera.

F Thin layer chromatography results

Because these investigations were exploratory, only the interesting results are outlined.

Amino acids in the hydrolysates were identified by the Rf values in comparison with marker amino acids and also from colours given with polychromatic ninhydrin. A subjective score of relative concentrations is given thus:-

-	not detected
+	trace only
++	distinct spot
+++	very pronounced spot

Amino acid	ripe male *fluviatilis*	ripe female *fluviatilis*
cystine	+	++
histidine	-	++
aspartic acid	++	++
lysine	++	++
glutamic acid	++	++
glycine	+++	+++
threonine	-	+
hydroxyproline(?)	-	+
tyrosine	++	++
alanine	++	++
proline	++	++
tryptophane	++	++
valine	++	++
methionine	++	++
leucine	++	++
isoleucine	++	++
phenylalanine	++	++
serine or arginine	++	++

Ripe males yielded 15 amino acids and ripe females yielded 18. These results are similar to those found by B.J.R.Taylor (pers. comm.). The differences suggest a field which may be worthy of more extensive examination.

IV DISCUSSION

A Identification

The original objective of this study was to provide a non-lethal method of identification of individual ammocoetes of the three British lampreys. As stated above (I E), all existing methods of distinguishing between ammocoetes of the two Lampetras are either not applicable to individuals or kill the animal.

As experimentation proceeded, it became clear that the identification of marinus ammocoetes was straightforward. Except in the case of small marinus ammocoetes, the existing method of identification by caudal pigmentation is as accurate as, and is quicker than, any immunological technique. At its present stage, the immunological method reported here is of little use with small ammocoetes. There is so little mucus available from such animals that the use of pieces of skin, and their almost inevitable death, is required to permit unequivocal identification.

L. planeri and L. fluviatilis ammocoetes have proved to be as similar in the antigenicity of their external mucus as in their external morphology. As shown in the results (p. 94 et seq.), the precipitin patterns produced by any one antigen material from the two lampreys are in most respects identical. Although differences did occur with antigens from both lampreys tested against the same serum, they were, with one exception, never consistent; and it is clear that the same system of antigens is present in the mucus of both planeri and fluviatilis. If any differences exist, other than the one dealt with below, they are probably quantitative and are confused or even masked, by the

Antigen	Antisera		
	anti-marinus	anti-planeri	anti-fluviatilis
marinus	7	7	7
planeri	0	6	0
fluviatilis	7 and/or 8	0	7 and/or 8

TABLE VII. The occurrence of the 'eyebrow' lines 6, 7 & 8.

Antigen	Antisera		
	anti-marinus B'	anti-planeri C'	anti-fluv. A'(B')
marinus B	BB'	BC'	BA' (BB')
planeri C	CB'	CC'	CA' (CB')
fluviatilis A(B)	AB'(BB')	AC'(BC')	AA' (BB')

TABLE VIII. Antigen/antibody crosses responsible for the eyebrow lines. A, B & C are the antigens and A', B' and C' are the antibodies to these antigens.

Antigen	Antisera					
	anti-marinus B'		anti-planeri C'		anti-fluv.A'(B')	
	expected	observed	expected	observed	expected	observed
marinus B	7	7	7	7	7(+7)	7
planeri C	0	0*	6	6	0(+0)	0
fluviatilis A(B)	8(+7)	8 &/or 7*	0(+6)	0	8(+7)	8 &/or 7

TABLE IX. Expected and observed results of crosses showing the eyebrow lines. (* are the result of M7 crosses.)

(Facing p. 100)

inherent variation of the patterns produced by occasional slight differences in the reagents and techniques used.

The one clear exception to the general agreement of patterns produced by fluviatilis and planeri mucuses is the result obtained using M7 serum (Figs. 69 & 70). An 'eyebrow' type line (No. 8) is produced by this serum with fluviatilis, but not with planeri, and this difference is consistent.

It is difficult to explain this result, but analysis of other crosses have helped to suggest its origin. The occurrence of the eyebrow lines, numbers 6, 7 and 8, is given in Table VII. In these crosses, 6 and 7 are classified by their occurrence in a particular antigen rather than their position, but many skin extracts gave both of these typical eyebrows and there are good grounds for believing them to be different in marinus and planeri.

To explain this distribution of eyebrow lines and thus the patterns given by M7 and certain anti-fluviatilis sera, it is necessary to make the following assumptions:-

i) there are three different, but closely related antigens responsible for lines 6, 7 and 8.

ii) marinus has one of these antigens - let this be B.

iii) planeri has another of these antigens - let this be C.

iv) fluviatilis has the third antigen, A, and also a little of the marinus antigen B.

v) The antigens are sufficiently alike that antibodies to them will react with the other antigens, except that antibody A' (to the A antigen) will not react with antigen C, and antibody C' will not react with antigen A.

vi) antigen B, as given in v) will react with antibodies A'

B' and C', but antibody to B (i.e. B') will not react with antigen C.

The reactivities of these antigens and their antibodies may be expressed thus:-

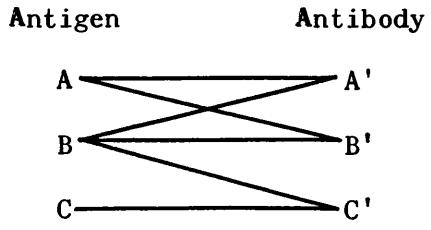


Table VIII gives the crosses possible. If antigen B gives line 7; C gives line 6, and A, line 8, the expected results and the observed results for comparison will be those shown in Table IX. The M7 crosses are those marked with an asterisk. Apart from the absence of a possible line 6 in the fluv./anti-plan. cross, the observed results agree with those predicted from the hypotheses i) to vi) above.

The dangers of implying a taxonomic relationship from an examination of only a small part of the total pattern are here apparent. On analysis of these eyebrow lines alone, it would seem that fluviatilis is more closely related to marinus than to planeri, an unlikely conclusion. It is possible that these antigens are affected by the anadromous life histories of marinus and fluviatilis, and may be an adaptation to the marine phase. If this were so, the greater similarity between marinus and fluviatilis in this respect, than between these two and planeri would be expected.

At the present time, it is not possible to use the difference expressed by M7 serum as a method of identification of individual animals. The quantities of mucus used in the micro-immunoelectrophoretic technique are very small indeed; about 25/ug (dry wt)

per slide, respresenting 5/ul of a 0.55% mucus solution and it is possible to scrape this quantity of mucus from a single ammocoete. However, besides the physical difficulties of handling such quantities, samples must be standardised to allow a valid comparison of results. Pooled mucus has been freeze dried and reconstituted to allow the use of comparable strength solutions, and raw mucus samples can be compared spectrophotometrically if sufficient quantities are available. It is difficult to envisage what method of routine standardisation could be employed with the quantities of mucus available from individual ammocoetes.

However, there is another approach to the problem; that of immune adsorption followed by the labelling of selected antibodies. As M7 serum discriminated between fluviatilis and planeri by the presence and absence of a single line, it should theoretically be possible to isolate the antibodies to this line for use as a specific serum. Such a serum would give a positive result with fluviatilis and negative with planeri. Used in conjunction with a similar specifically selected anti-marinus antibody, even the smallest ammocoete could be positively identified.

Various techniques of specific antibody isolation would be applicable; any method where planeri mucus would be adsorbed onto an inert porous material would be suitable. M7 serum, passed through this material would then lose all of its planeri antibodies and the serum collected would contain only those anti-fluviatilis and anti-marinus antibodies not common with planeri. Ideally, anti-marinus antibodies, other than those responsible for line 8, and all of the other serum components should be discarded. This

could be achieved by the passage of the serum through a material bound with fluviatilis mucus, when the anti-fluviatilis antibodies would remain attached to their antigens on the 'filler'. These antibodies may then be eluted from the 'filler' by a change in pH and the resulting specific anti-fluviatilis amtibody solution collected and reduced to give the requisite concentration.

An identification test should be quick and simple to use as well as being reliable, and for these reasons it would be suitable to label the anti-fluviatilis antibody with a fluorescent compound. A variety of simple labelling techniques are available.

A smear of mucus on a microscope slide should be sufficient to test the identity of any ammocoete. It would only be necessary to place a drop of the labelled antibody on the air dried smear and incubate at room temperature for about 30min (this period may be considerably reduced by experience). After several washes with buffered saline, examination under ultra-violet light should reveal those smears with which the antibody has reacted and thus those ammocoetes which are fluviatilis. The major practical difficulty in the application of such a smear test would probably be the segregation of the individual ammocoetes tested, while the incubation is proceeding.

Theoretically therefore, the immunological methods used in this study could be developed to yield a useful identification method. Practically however, there are several difficulties, although these are not insurmountable, given time and perhaps some luck.

i) Quantities of antiserum needed.

The only antiserum of practical use so far, comes from a single rabbit. To isolate antibodies to a single antigen in

useful quantities it would be necessary to adsorb a considerable quantity of serum. Using the cyanogen-bromide method, 1mg of antibody has been isolated from 1ml of serum with a titre of this antibody of 1 in 32. The titre of the particular anti-*fluviatilis* antibody (anti-8) in M7 is considerably lower than this (1:2 or even less). The antigen/antibody system involved is one of the weaker ones unfortunately.

It is probably impossible to obtain the volume of antiserum necessary from one rabbit. Although immunisation with F.C.A. results in a sustained response to minimum quantities of mucus, a certain degree of specificity is lost. If, as has been necessary in this study, further immunisations are needed, the specificity is further reduced.

It is in this problem of suitable rabbits that luck could perhaps play a part. M7 was the only rabbit out of over 20 immunised that was of use, but of these 20+, only seven were immunised against *marinus*. Anti-*marinus* serum is the only serum that will detect the probable eyebrow line differences discussed above. Out of these seven anti-*marinus* rabbits, only three were good antibody producers in that they gave a relatively high titre serum showing many lines when crossed with *marinus* antigen.

With a sample of three such rabbits, it is impossible to know whether the occurrence of one rabbit giving a serum like M7 is a true reflection of the distribution of rabbits sensitive to the difference between *planeri* and *fluviatilis*. It was possibly lucky to get one such rabbit out of three, but it is equally possible that most rabbits can discriminate and that it was unlucky to get two that could not. The only way of investigating the

distribution of M7-type rabbits would be to immunise and test a much larger sample.

ii) Quantities of antigen needed.

Immunisation of a sufficiently large number of rabbits would need much greater quantities of marinus mucus than have so far been used; although only small quantities for maintaining the titre within selected rabbits would be needed after the initial immunisation.

The availability of marinus adults varies from season to season, but fortunately they are good mucus producers. 0.625g (dry wt.) of mucus would be needed to immunise 25 rabbits using F.C.A. and it should be possible to collect most of this in a good season.

Five or six rabbits, discriminating between planeri and fluviatilis by the same criterion should provide sufficient serum. If, as in M7, the discrimination is by the presence of a line in fluviatilis, and its absence in planeri, planeri mucus would be required to adsorb out son-specific antibodies. It is very difficult to calculate how much adsorbant mucus would be needed, not knowing the exact titre of each antibody in the serum, nor the ratio of adsorbant to antibody required for an optimum reaction. Extrapolating from the cyanogen bromide method used with a high titre anti-human IgG serum and human IgG, between 3.0 and 6.0g of mucus would be required to adsorb out the anti-planeri antibodies from 300ml of a serum with these antibodies at a titre of about 1:32. 300ml of adsorbed antiserum would yield about 30mg of specific anti-fluviatilis antibody, with this at an original titre of 1:2, after separation on fluviatilis mucus.

Quantities smaller than this would be difficult to label. These figures are estimates and may be inaccurate in either direction.

Even if as little as 1g of planeri mucus is needed, it is unlikely that this quantity could be collected by present methods. The yield per individual large ammocoete or adult planeri was about 0.3mg (dry wt.). It is possible that a method of stimulating mucus production can be found but the number of animals required is still too great.

A more fruitful approach would be the use of skin extracts. These are powerfully antigenic and if a physical separation of their antigens could be achieved, chosen antigens could be used as specific adsorbants. Preparative electrophoresis on agar has not so far given a suitable separation but this is probably because the variables involved have not yet been fully explored.

Once sufficient antibody has been isolated, fluorescent labelling should be straightforward. It would be necessary to establish the degree of auto-fluorescence of the unlabelled mucus, and if this is unacceptably high, an alternative label such as ^{125}I could be used.

Given sufficient time, and a good supply of both rabbits and lampreys, this method is capable of development into a useful identification technique. Fortunately, labelled sera and labelled antibody solutions need be used in small quantities in very dilute solutions. 50mg of labelled antibody should be sufficient to test between 1,000 and 5,000 mucus smears.

B Lampreys and speciation

1 'Paired species'.

The immunoelectrophoretic techniques described could be used to identify ammocoetes and it is now relevant to consider the results obtained with regard to lamprey relationships and their taxonomic status.

None of the sera gave any positive results with Teleost antigens. This is the expected result in view of the accepted ancient origin of the lampreys.

P. marinus has been placed in a different genus from Lampetra planeri and L. fluviatilis, and the immunoelectrophoresis of the external mucus supports this classification and the probably long divergence of Petromyzon and Lampetra stocks.

L. planeri and L. fluviatilis seem very closely related on the basis of these results. They are usually recorded as paired species, but the exact taxonomic status of these two, and of the other paired species, has long been argued. Zanandrea (1959 b) was the first to use the term 'paired species' to describe those lampreys where a non-parasitic form is morphologically identical with, or very similar to, a parasitic form except in adult size. The members of most species pairs have the same distribution, and often occur together in the same river system.

Ten parasitic species have given rise to non-parasitic derivatives and Ichthyomyzon bdellium has probably given two; Lampetra japonica being ancestral to three. There are also various 'unattached' non-parasitic species which usually show certain 'degenerate' features.

The morphology of paired species, differences and similarities between them, and their taxonomic status have been discussed by, among others, Leger (1924) who also reported views of several earlier authors; Hubbs (1925); Hubbs and Trautman (1937); Weissenberg (1925, 1926 & 1927); Cotronei (1927a & 1927b); Enequist (1937); Young (1950); Zanandrea (1954, 1958b, 1959b+c & 1961); Vladykov (1958); Vladykov and Follett (1958 & 1965); Hardisty (1961a, 1963 & 1964); Privol'nev (1964); Potter, Lanzing and Strahan (1968) and Hardisty, Potter and Sturge (1970).

The origin of a non-parasitic species has usually been ascribed to paedomorphosis, neoteny or other processes of precocious gonadal development in the original parasitic population (Leach, 1940 & 1951; Young, 1950 & 1962; Zanandrea, 1956 & 1961 and Hardisty, 1960, 1963, 1965 & 1970). Origin by any of these processes would so advance sexual maturation that maturity follows closely on metamorphosis, the adult life being reduced to a few months only. Zanandrea (1961) envisaged this increasingly early maturation as taking place during a sequence of life history changes from fully anadromous (L. fluviatilis-type) through a landlocked migratory stage (landlocked P. marinus-type) and a freshwater parasitic stage (Ichthyomyzon unicuspis-type) to the brook lamprey type with the complete elimination of the adult feeding phase.

As paired species are so widespread, it is surprising that neotenous or paedomorphic lampreys are so rarely found if this were the mode of origin of the non-parasitic species. Zanandrea (1956 & 1957) described 12 neotenous female ammocoetes of

L. zanandreai from the Po Plain in Italy, but this is the only reported case of neoteny in lampreys and no males have been reported.

Recent work by Hardisty (1961b & 1970), Hardisty, Potter and Sturge (1970) and Hardisty and Huggins (1970) on the growth rates and duration of larval life of planeri and fluviatilis has suggested a different interpretation of the origin of non-parasitic lampreys. It is probable that both planeri and fluviatilis have a total life duration of seven years and that the transition from the parasitic fluviatilis to the non-parasitic planeri type of life history has been by a relative lengthening of the larval period. In fluviatilis the length of larval life is about 4½ years and in planeri it is about 6½ years. In both cases the final larval year is probably a non-growing 'resting year', because metamorphosing-length ammocoetes can be found throughout the year. A 'resting year' has been suggested for some North American species by Gage (1928) and Leach (1940); and Potter (1970) has found evidence for a non-growth period in Mordacia.

The theory of the origin of non-parasitic lampreys by relative lengthening of the larval period explains the larger size of the non-parasitic lampreys at metamorphosis. It is hard to envisage an alternative theory of precocious sexual maturity that would account for this size difference, or for the undoubtedly longer period of larval life in planeri than in fluviatilis.

However, although the theory of delayed metamorphosis (and therefore lengthened larval life) explains the process involved in the origin of brook lampreys, it does not of itself

suggest a reason for the derivation of non-parasitic types, nor does it clarify the taxonomic status of the 'paired species'.

2 Species definitions and concepts.

Speculations on the speciation of planeri from fluviatilis, their present relationship and their taxonomic status, can only be useful if the concept of 'species' is meaningful and if the generally accepted theories of speciation have been explored. Unfortunately, species concepts and speciation are themselves largely meaningless and irrelevant in this context unless they are related to lampreys, and the discussion therefore tends to become circular. However, as the 'species' is the most basic, although the most elusive, unit in taxonomy, a brief discussion of species concepts is perhaps the most fruitful point at which to enter the circle.

Of the word 'Species', Huxley (1942) wrote, "....logic demands that we should define the term. It may be that logic is wrong, and that it would be better to leave it undefined, accepting the fact that all biologists have a pragmatic idea at the back of their heads. It may even be that the word is indefinable."

It is true that the word 'species' is meaningful to most biologists, but that there have been as many definitions of species since Linnaeus as there have been systematists. "There is perhaps no other subject in biology for which one can document as long standing a controversy as the species concept" (Mayr, 1969).

Part of the controversy has always been whether or not species are real entities, or only man made and more or less arbitrary divisions of the "seemingly chaotic multiformity of nature" (Dobzhansky, 1937). Mayr (1969) quotes Bessay (1908) as "Nature produces individuals and nothing more....Species have no actual existence in nature. They are mental concepts and nothing more...Species have been invented in order that we may refer to great numbers of individuals collectively." This point of view was possibly useful when species were defined on a purely morphological basis, with an underlying typological concept, but it must be untenable to the majority of present day biologists. Not only is there ample evidence of the existence of discrete genetic groups in nature, but, as Dobzhansky (1937) pointed out, species are a necessity. "....Maintenance of life is possible only if the gene patterns whose coherence is tested by natural selection are prevented from disintegration due to unlimited hybridization." Most organisms are well adapted to their environment and can only remain so by segregation from other organisms whose adaptations have been produced in response to a different environment.

The morphological species concept is the oldest scientific view of species and is based on the definition "....a species is a group of individuals or populations with the same or similar morphological characters." (Mayr's interpretation in 1942). From the time of Linnaeus until Darwin's views became widely accepted, the morphological species concept gave the only workable definition of a species. It embodies an older concept, 'essentialism', in which observed diversity reflects the existence of

a finite number of underlying types. Variation is due to the imperfect manifestation of the types, which do not stand in any particular relationship with each other. This essentialist view was more or less expressed by Plato (the eidos) and Aristotle, and was the underlying philosophy of the morphological species concept.

Most early taxonomists used dead specimens, with little or no information on their ecology or behaviour, and under these circumstances a morphological concept of species was the only workable one. However, even Linnaeus was not a purist in this respect; he originally classified drake and duck mallards as separate species, which on strictly morphological grounds they might well be, but he later reclassified them correctly on gaining further information on their biology.

The morphological species concept has not been entirely superseded. It provides the only useful way of classifying groups where individual specimens only are available, or where the biology of a group is not sufficiently well known. As Mayr (1942) wrote, attempts have been made to update the morphological species concept and he quotes Wilhelmi (1940)..."Species of helminths may be defined tentatively as a group of organisms the lipid-free antigen of which, when diluted to 1:4000 or more, yields a positive precipitin test within 1 hour with a rabbit antiserum produced by injecting 40mg of dry weight lipid free antigen material, and withdrawn ten to twelve days after the last of four intravenous injections administered every third day." This is, in fact, a morphological species definition, and is possibly even less useful than the more traditional

approach where the morphology of the whole animal was considered.

The drawbacks of a purely morphological species concept are fairly obvious. Where a species shows considerable morphological variation, either as sexual dimorphism or as different stages in the life history, each form is likely to be classified as a different species. The best known example is that of the leptocephalous larva of the eel, but larval lampreys were also classified as a different group from the adult on morphological grounds until their biology was better known.

Morphological classification is likely to be inaccurate also, when applied to 'sibling species', those species that are extremely similar morphologically, yet have different biologies and may not even be closely related.

The modern concept of species - the biological species concept for want of a more descriptive term, is as elastic as the morphological species concept was rigid. The principal theme of the biological species concept is that of the reproductive relationship of populations. Thus a species is defined by its non-interbreeding with other populations. ".....to be a different species is not a matter of degree of difference but of relational distinctness." (Mayr, 1969). This concept is probably as close to the reality of species as it is possible to get, but to define species by these criteria and to apply such a definition to actual groups of organisms is very difficult. Mayr (1942) wrote that trying to produce a biological species definition was like "...trying to establish a fixed stage in the evolutionary stream. If there is evolution in the true sense of the word....we should

find all kinds of species - incipient species, mature species, and incipient genera, as well as all intermediate conditions. To define the middle stage of this series perfectly, so that every taxonomic unit can be certified with confidence as to whether or not it is a species, is just as impossible as to define the middle stage in the life of man, mature man, so well that every single human male can be identified as boy, mature man, or old man."

Dobzhansky (1937) defined a species "as that stage of the evolutionary process at which one actually or potentially interbreeding array of forms becomes gegregated into two or more separate arrays which are physiologically incapable of breeding." Mayr (1942) pointed out that this is a description of the process of speciation, and that species are the result of the process and not the process itself. His definition was that "species are groups of actually or potentially interbreeding natural populations, which are reproductively isolated from other such forms." As a definition this presents as many problems as it solves; there is no way of assessing the potentiality of interbreeding between geographically separate populations, and many of the isolating mechanisms that serve to prevent interbreeding in nature are disrupted under the conditions of captivity.

It is difficult, therefore, to classify by this type of definition, any populations that do not live in adjacent or overlapping ranges, or whose biology is not well known. The biological species concept is fruitful and worthwhile, as it is based upon the reproductive relationships of populations, which are themselves reflections of the genetic nature, and therefore phylogeny and adaptations of the populations.

Finally, the most useful of species concepts utilises both morphological and biological data, and although at first sight seems meaningless, it is in fact the concept by which most groups are classified. Darwin in 1859 wrote "In determining whether a form should be ranked as a species or a variety, the opinion of naturalists having sound judgement and wide experience seems the only guide to follow". Or, as Mayr (1942) put it, "A species is a systematic unit which is considered a species by a competent systematist".

It is perhaps too much to try and find a definition of species that is both concise and all embracing. There are too many exceptions to whatever criteria are applied, there are too many borderline cases, and there are too many populations where the necessary comparisons cannot be made. An understanding of the nature of species, what they are and how they are formed, and why, is what is really necessary; and attempts at definitions as such will not add to this understanding. "Disputes as to what constitutes a species are fruitless, 'a species is a thing described as such'. This is simply a matter of definition. If on grounds of expediency one definition is preferable to another, it may be well to urge its general adoption. But its adoption or rejection neither add nor subtract one jot from our stock of ascertained fact". (Lotka, 1956).

3. The reasons for speciation

Except in cases of rapid environmental change, often caused by human interference, species are in equilibrium with their environment. This equilibrium is the result of the potentially infinite increase of any population meeting the resistance of limiting environmental factors. "In looking at nature, it is most

necessary.....never to forget that every single organic being may be said to be striving to the utmost to increase in number, that each lives by a struggle at some period in its life, that heavy destruction inevitably falls either on the young or the old, during each generation or at recurrent intervals. Lighten any check, mitigate the destruction ever so little and the number of the species will almost simultaneously increase to any amount" (Darwin, 1872). The potential increase is usually kept within quite restricted limits. Lack (1954) found that bird populations fluctuate in numbers with the highest number being usually only two to five times that of the lowest.

The environmental factors that restrict unlimited increase can be classified as follows (as suggested by Andrewartha & Birch, 1954):-

I. Climate.

II. Food.

III. A place in which to live.

IV. Other animals of the same species.

V. Other organisms of different species.
   a) non-predators.
   b) predators.

All of these environmental factors will impose limits upon the species, both as to numbers and distribution.

Climate will usually impose the limits of distribution and may, in some cases, also restrict the actual numbers within a species. It is unlikely however, that climate is usually the chief limiting factor, as it is not 'density dependent' (Haldane, 1954). Any environmental factor that tends to maintain the status

quo must be density dependent, i.e. its effects must be more severe at high densities of population than at low. Any factor, such as climate, that is not related to actual numbers must inevitably lead to unstable fluctuations in numbers and if sufficiently severe in its action it could lead to the extinction of the species. All the other features of the environment listed can act as density-dependent factors.

Food is considered to be the chief limiting factors at all trophic levels except herbivores, by Hairston, Smith and Slobodkin (1960). This is based on the observation that fossil fuel accumulation throughout geological time has been negligible, compared with the rate of energy fixation by photosynthesis. Almost all of the energy trapped therefore, must flow through the biosphere and be lost; and so all organisms taken together must be energy limited. Any particular level which is not food limited must, of course, be held below the food resource level. Hairston et al,(1960), concluded that only herbivores are not food limited because they rarely deplete their food, at least so much that starvation becomes the limiting factor. Exceptions to this have occurred when predators of herbivores have been removed by man and overgrazong has resulted. Thus herbivores, and herbivores only, are predator limited.

While this view is probably an oversimplification it is true that many species are food limited, both in distribution and numbers. A 'place to live' is a limiting factor in territorial species, and in those that need places to burrow &c.

Territorial behaviour may be an adaptation to sharing of limited food resources. The acquisition of a territory ensures sufficient food for that individual. Lack of enough 'places in which to live' will restrict animal numbers and also restrict range. Predation, especially parasitism, is a limiting factor in a few species, parasitism being especially important at high population densities.

Whenever a species is restricted by a limited resource, competition will take place (see Meaning I of Birch, 1957 for a more detailed discussion).

Crombie (1947) stated that the proportion of organisms being eliminated by competition will vary inversely with the severity of the agencies such as climate or predation, that reduce the ratio of population to resources. If most species are food limited (Hairston et al, 1960), then intra- and inter-specific competition must usually be intense.

Existing species are 'hemmed in' by their environment, either by physical factors such as climate, or through intra-specific competition for limited resources. New species arise in response to these limitations imposed by the environment. A change occurs that allows the organism to more successfully overcome the environmental barrier. It will either avoid competition with the parental species or become more successful in the competition. If, for instance, the adaptation is to climate, a physiological adaptation may emerge affording the needed advantage. For example, an insect species develops a diapause mechanism that allows it to overwinter successfully in a harsher climate than it could previously tolerate. The new

species will be able to exploit a new environment. If the adaptation is such that the new species is more successful than the old, it will tend to oust the old species.

4. Methods of speciation

Dobzhansky (1940) and Mayr (1942) differentiate between the two stages necessary for speciation to occur. The first is the establishment of diversity and the second is the development of discontinuities. White (1959) stressed that "...the really significant thing in the origin of a new species is the building up of genetic isolating mechanisms...", i.e. the development of discontinuities.

Both stages are necessary for speciation and the second is also necessary for the maintenance of the integrity of the new species should it recontact the parental species.

This section of the discussion will consider speciation in the four sections:-

a) the origin of diversity
b) isolating mechanisms
c) isolation
d) rate of speciation.

a) the origin of diversity

All populations, species included, show some degree of variation. Much of this is environmentally induced and as it is non-hereditable, plays no part in evolution.

It is difficult to assess the degree of variation in any species. Dobzhansky (1951) pointed out that most variability

in wild populations is concealed as the genes are recessive or lethal - "One may nevertheless suspect that the enormous stores of potential variability found in Drosophila should be paralleled in other organisms". Mayr (1963) suggested that there may be up to 400 mutational sites possible on one gene, the mutations ranging from lethality to non-detectability.

However, as Mayr (1969) pointed out, it must always be remembered that no organism is a 'bag full of genes'.

Every species is an adaptive complex which fits a certain niche and the adaptive value of this complex is not determined by a few genes but by the phenotype as a whole (Dobzhansky, 1940). All of the genes interact, each modifying the total environment of all of the other genes (Mayr, 1942). Most species show remarkable phenotypic uniformity over considerable distances, such that gene flow between the parts of the population seems to be an inadequate explanation. Mayr (1969) suggested that this uniformity is because of the essential cohesion of the genotype; all the populations of a species will share the same 'epistatic interactions' brought about partly by the possession of the same pleiotropic genes (Dobzhansky, 1951), i.e. those genes that affect more than one character, and partly because the possible canalizations of adaptive changes are the same. "....the populations of a species seem to share the same homeostatic systems and the same physiological constants as a consequence of the same basic genotype" (Mayr, 1963). It is for this reason also, that closely related species tend to show the same type of adaptation towards any given environmental factor.

Diversity must therefore be viewed against the background of those coherent, rather stable, gene complexes.

Simpson (1953) classifies the sources of diversity as follows:-

I. Recombination
  i) of genes
  ii) of chromosomes

II. Mutation
  i) of genes
  ii) of chromosomes
    a) structural
    b) numerical

Of these, only mutations can actually provide new genetic material. The 'recombinations', ordinary Mendelian inheritance of alleles, and chromosomal recombination by gametes with unlike chromosomes, probably provide the basis of intraspecific variations such as polymorphism and geographical adaptation.

A species is polymorphic if it shows consistent discontinuous hereditable variation; but as Dobzhansky (1951) pointed out, "Polymorphism is a loose descriptive term; all Mendelian populations are more or less polymorphic". He considered that polymorphism is adaptive and is maintained when heterozygotes show adaptive superiority over the homozygotes and that it is widespread in the living world.

There are many examples of polymorphism, some of which seem to show a clear correlation with adaptation. Timofeeff-Ressovsky (1940) showed that the beetle, Adalia pipunctata, has red and black adults, both the result of simple genotypes. Overwintering by the adults produces a mortality of 96% in the black adults but only 89% in the red; whilst over the summer, the black form increases rapidly to become 59% of the population by the Autumn.

Drosophila pseudobscura has three different gene arrangements in California - Chiricahua, standard and arrowhead -. The relative frequency of the standard falls between March and June whilst that of Chiricahua increases. During the hot season of the year, June to September, the increases are reversed. Arrowhead changes are less regular but largely parallel those of Chiricahua (Dobzhansky, 1943). This pattern is repeated annually with minor variations. In drought years, arrowhead increases to be overtaken by standard during wetter years.

Cain and Sheppard (1954) questioned Dobzhansky's theory of adaptive polymorphism on the grounds that polymorphism is maintained by selection and not necessarily by adaptation.

Geographical races, where thoroughly investigated, have been shown to be adaptive. The difference between this phenomenon and polymorphism is that the geographical races show continuous variation along a series although the gradations between populations may be steep. Polymorphism is probably the result of combinations of a few genes, each showing comparatively major effects, whilst geographical variations or races, are the results of recombinations of series of genes, each with small additive effects.

Moore (1949 & 1950) reported geographical races of Rana pipiens from Canada to Panama, the continuous variable being the optimum temperature for egg development. The most northerly races (in Vermont) had the lowest optimum temperatures whilst races from Florida had the highest. A population in the high mountains of Costa Rica, where temperatures were generally low,

showed a lower optimum temperature, nearer to those of Vermont than Florida. Hybrids between the races showed defects which were most severe between the Vermont and Florida races; the Costa Rica population being more compatible with the northern races than the southern races.

Large scale geographical variability produces 'clines' (Huxley, 1939), a series of populations usually with an extensive distribution, showing continuous variation of one or more variables. Examples of such clines are those of the great tit, Parus major, and the herring gull, Larus argenteus (Mayr, 1942). Both of these birds have end species which are so different that they do not interbreed, although there is genetic continuity between them through the intermediate populations.

It has long been assumed that goegraphical races and polymorphic populations are potential spcies. Dobzhansky (1944) wrote, "....the principle, the validity of which is securely established, that species evolve from races....." It is true that species usually arise from races but it must be emphasised that the essential difference between them is reproductive isolation. "Races are genetically open systems. Their populations are channels through which genes can and do flow from race to race". Speciation necessitates the closure of this system by isolating mechanisms; possibly by a few genetic or chromosomal changes, or perhaps by the complete restructuring of the population (Mayr, 1969).

As such a closure is necessary, simple isolation of a geographical race or part of a polymorphic population, will not give a new species. All the genetic material and sources of variab-

ility are held in common with the parent population, and it seems unlikely that mechanisms to prevent interbreeding could arise from this material. Such mechanisms would, after all, be disadvantageous to a continuous population and combinations tending to produce them would be selected against, although recombinations can produce adaptations.

It is necessary therefore, to look at Simpson's (1953) category II (see p. 121) of variation for those factors that may produce genetic isolation, and therefore the potential for speciation.

Genetic mutation (IIi) is self explanatory. Chromosome mutations (IIii) include duplications, translocations and inversions being structural changes (i.e.IIiia) and changes in karyosome number such as polyploidy (i.e.IIiib).

The cytological causes of most mutations cannot be easily investigated; a phenotypic change resulting from mutation may be caused by a gene mutation or a chromosomal mutation. The subsequent history of a mutation however, may be greatly affected by its cause. A single gene mutation will obey the rules of Mendelian inheritance and especially if it is recessive, it will be absorbed into the parental genotype. If, as is likely in a well adapted species, it is disadvantageous, it will be selected against. Chromosomal mutations may also be 'swallowed up' by the parental genotype. Where changes in the number of chromosomes is involved however, as in polyploidy, the splitting of a metacentric chromosome into two acrocentrics, or the fusion of two acrocentrics to give one metacentric, fusion between gametes

with the new number and parental-type gametes is likely to be defective (White, 1957).

As mutations can produce new genetic material and therefore the potentiality of isolating mechanisms, it is from these events that the major specific differences will arise.

b) Isolating mechanisms

The second, and perhaps the most crucial stage of speciation is the establishment of discontinuities between populations that are diverging. "...the essential feature of the process of species differentiation is the formation of discrete groups of individuals which are prevented from interbreeding with other similar groups by one or more isolating mechanisms. The expression 'isolating mechanisms' seems to be a convenient general name for all the mechanisms hindering or preventing the interbreeding of racial complexes or species." (Dobzhansky, 1937).

Although a new type of population may form through the accumulation of adaptive genetic differences, it will not necessarily speciate. To become a good species a population must acquire genetic or chromosomal mechanisms that will prevent gene flow between it and any other species.

Isolating mechanisms are distinct from the accumulation of differences and adaptations that enable the population to fill a new niche and they may be disadvantageous to the original parental population should they arise prematurely.

Dobzhansky (1940) wrote that isolating mechanisms are a fundamental common property of species and that they/so "...is

Lee (1967) & Lewis (1969)	Grant (1963)	Mayr (1942)	Dobzhansky (1951)	Dobzhansky (1937)
A. pre-fertilisation.	I. Spatial	a) Restriction of random disperal	1) Geographical isolation	i) Geograph. isolation
	II. Ecological		2a) Ecological isolation (habitat)	ii) Ecological isolation.
			b) Temporal isolation	
	III. Reproductive	b) Restriction of random mating	c) Sexual, psycholog.[1] ethological isol.	iii) Sexual isolation.
			d) Mechanical isol.	iv) Mechanical isolation.
B. post-fertilisation.		c) Restriction of fertility	e) Gametic isolation.	
			f) Hybrid (F_1) inviabil.[y]	v) Hybrid (F_1) inviability
			g) Hybrid (F_1) sterility	vi) Hybrid (F_1) sterility
			h) Hybrid (F_2) breakdown.	vii) Hybrid (F_2) breakdown.

TABLE X. A table of classification of isolating mechanisms.

(Facing p. 127)

the new complex may even be deleterious during part of the life history, but if they are advantageous at another stage or necessary to preserve the balance of the whole complex, they will be incorporated. It is from these peripheral changes, not directly environmentally adaptive, that most isolating mechanisms probably arise, as incidental by-products of genetic divergence (Mayr, 1963). This must certainly be so in the case of 'sterility genes', especially if such genes are separate from 'physiology and morphology genes', as Dobzhansky (1940) believes, as they would be highly disadvantageous in a coherent population.

As far as is known, all isolating mechanisms, with one exception, are the result of changes in the genetic complex, rather than single genetic or chromosomal mutations. The exception is polyploidy, but this is the only case where a single change could give complete, single step reproductive isolation.

There have been many classifications of types of isolating mechanisms; the major ones are given in Table X. The various categories arranged by the different authors are all meaningful but in different respects. Dobzhansky (1937) listed the actual mechanisms whereby gene exchange was prevented and Lee (1967) makes the point that only pre-fertilisation barriers are really efficient.

Geographical isolation, an element of Mayr's (1942) 'restriction of random mating' operates either with geographic or spatial isolation, where the populations occur in different territories. It is not a true isolating mechanism, as it is obviously not intrinsic to the species. Remove the geographical barrier and unless other mechanisms have developed, 'introgression' (Bigelow,

1965) will occur and the populations will merge. 'Spatial separation' is distinct because, of itself, it has no genetic basis and consequently no relevance to taxonomy even though it may set the stage for evolutionary divergence and speciation (Lewis, 1969).

Ecological isolation, where breeding populations occur in different habitats, or breeding occurs at different times, is genetically based and it is a fairly common isolating mechanism. Dobzhansky (1937) gives the example of Gasterosteus in Belgium, the two populations of which are isolated by both habitat and breeding season. Although hybrids can be artificially produced, they appear rarely in nature.

The two species of field crickets, Acheta pennsylvanius and A. veletis, described by Alexander and Bigelow (1960) are isolated apparently by time of breeding alone and this is the result of different overwintering stages. Ecological isolation alone is a rather uncertain isolating mechanism; if habitat differences separate two populations there is always the chance of hybrids where the two habitats meet. Similarly, hybrids may often occur if the breeding seasons are not sufficiently separated. By definition, if ecological isolation is the only isolating mechanism, hybrids will be fully fertile and viable and there will be no further barrier to gene flow.

Sexual isolation, i.e. weakness or lack of attraction between males and females of different species, was illustrated by Dobzhansky (1937) between races of Drosophila pseudobscura, and between D. pseudobscura and D. miranda. Its basis is often behavioural although this is not always possible to investigate.

Closely related frogs of the genus Heleioporus were reproductively isolated by the selection of species-specific male mating calls by the female (Lee, 1967).

Mechanical isolation is occasioned by the non-correspondence of genitalia physically. Many closely related insect species show clear differences in the genitalia although they are otherwise morphologically similar. These genital differences may well be the primary isolating mechanism in these species.

All of the isolating mechanisms so far mentioned are classified by Mayr (1942) as either restricting random dispersal or as restricting random mating. They are all pre-fertilisation mechanisms, so gametes are not wasted.

The other isolating mechanisms, the post-fertilisation types, can be classified as a series of viabilities. At one end of the scale is Dobzhansky's 'genetic isolation' when the spermatozoa of one species are not attracted to the ova of another, often because of chromosomal discrepancies or 'meiotic drive' (Sandler and Novitski, 1957), or are poorly viable in the sexual ducts. At the other end is 'hybrid breakdown', a loose term used by Dobzhansky to describe sterility , inviability or adaptive inferiority of F_2 generation crosses, or back-cross hybrids. For such hybrid breakdown to be isolating, gametes must be interfertile and F_1 hybrids viable and at least partly fertile. In such a situation there is a danger of introgression and blurring of the species limits. More commonly, F_1 hybrids, if produced at all, are poorly viable, or at least one sex is sterile.

Although interspecific sterility is probably the commonest and certainly the best recognised isolating mechanism, its origin is uncertain. Fisher (1930) pointed out that if hybrids between

two groups are infertile, genes tending to decrease future cross-matings will be selected, and later generations will tend to mate intra-group rather than inter-group. However, this assumes primary hybrid sterility. If A and B give a sterile hybrid, how can B be derived from A? (Sturtevant, 1938). Such sterility can only be arrived at by stages, with the initial hybrids being more fertile than later ones. "This infertility must be supposed to increase up to complete sterility - yet at first sight it seems that a decrease in fertility is precisely what selection cannot do" (Sturtevant, 1938). Within the hybrid group itself, selection is going to increase the fertility.

It is unlikely that hybrid sterility is the initial isolating mechanism during speciation by any process except polyploidy, and its origin is probably similar to that of other isolating mechanisms discussed below.

Most species investigated have several isolating mechanisms. If two good species are established in the same geographical area, it is axiomatic that they are utilising different resources. This being so, many species are ecologically isolated to some degree, but the ecological isolation is almost always reinforced by ethological mechanisms especially where the species are closely related, or have similar ecologies. Most species of passerine birds show ecological isolation of some degree, strongly reinforced by ethologically based isolation, e.g. closely related species show distinct plumage patterns and have characteristic calls. Primary ecological speciation of Lee's anurans (1967) is by choice of spawning situation, but pre-mating isolation is achieved by the species-specific calls of the males. Reproductive isolation is often ensured by several mechanisms, each rather

weak in itself, but when taken together complete isolation is ensured.

How isolating mechanisms evolve and become established throughout a new species has been the subject of much discussion. The two theories are either that isolating mechanisms arise during the period of isolation of the emergent species, or that they arise adaptively when the incipient species again come into contact. Although both of these views have flaws, they are not really incompatible and they are probably two stages in the acqisition of an isolating mechanism.

The first theory states that 'two populations that are completely isolated geographically will, in the course of time, diverge genetically so that when they are brought together again... they will be found to be genetically isolated in various ways' (White, 1959).

Isolating mechanisms arise as incidental by-products of genetic divergence, and hybrids must therefore be either sterile or inviable. In general, this theory is probably correct, but there are drawbacks when it is considered in detail.

Dobzhansky (1940) pointed out that the accumulation of genetic changes does not necessarily induce isolation. He quoted experiments with Drosophila in which races differing by more than a dozen genes have been synthesised, without impairing interfertility. He added that this is not proof that strains differing in hundreds of genes would show no limitations on cross-breeding. Bigelow (1965) stated that "Mechanisms that inhibit interbreeding could evolve incidentally during geographic isolation to a level

that would inhibit, or even prevent, interbreeding even between otherwise genetically compatible populations, and such mechanisms might maintain effective reproductive isolation if contact should be regained." Such isolation would, however, be precarious as they would not necessarily involve fundamental genetic incompatibility. If the isolation should begin to break down as is possible as the result of mutation, there would be a growing flood of genes into both populations from the other and the isolation would be erased (Bigelow, 1965).

The main drawback however, is the cost to the population of selecting any particular trait, and this cost may be unbearably high if the trait is not adaptive. Species gene pools are harmonious wholes and a new species must reorganise its gene pool containing the new adaptations such that it remains harmonious.

The cost of selection of any one allele is very high according to Haldane (1957). The deaths of a number of individuals equal to thirty times those in one generation are needed to substitute one gene for another at a single locus. If this is so, gene changes that are not adaptively superior are disadvantageous to the population as a whole and especially so if they disrupt the harmony of the whole gene pool.

It is difficult to see therefore, how isolating mechanisms, that almost always require more than one gene change, could arise within a population, spread and become fixed when they are not of adaptive value at that time. Development of isolating mechanisms are likely at some time to be disadvantageous to the isolated population. For example, if a parental population breeds

in June and the isolate acquires a gene giving August breeding, most of the population will still be June-breeding and the August-breeding individuals will be at a disadvantage. The principle exemplified applies to any isolating mechanism in general.

The second theory of how isolating mechanisms became established is that of Wallace (1959). He suggested that hybrids between the two populations would have a lower viability and elimination of these would lead to the survival of genotypes that only breed truly. However, the remarks above on hybrid sterility are relevant here; there can be no primary hybrid sterility between parent and daughter populations and natural selection will increase fertility, not sterility, wherever it acts. Secondly, Mayr (1963) showed that back-crossing and introgression of any hybrids will lead to a weakening of the isolating mechanisms.

The real origin of isolating mechanisms is probably a combination of these two theories. Any population, isolated from its parental population will begin to diverge from it. Mayr (1963) gave three main reasons for this divergence. Firstly, no two environments are exactly alike and as the isolate is now genetically independent of the conservative effect of the main gene pool, it can respond fully to the new selection pressures. Secondly, the isolate will not have the same genetic constitution as the parental population. Most isolates are small in numbers compared with the original total and will contain only part of the total parental genetic variation. This means that some rare genes may occur at high frequencies; that homozygotes rapidly become more frequent and that by the Sewall-Wright effect, many genes may be totally lost.

Thirdly the bases of evolution will not proceed in parallel in the two populations. The probability that the same mutations and recombinations would occur at the same time in both populations is very remote.

As the divergence increases, isolating mechanisms may arise but because they are adaptive *in situ*, not in the pre-adaptive sense of their usefulness as isolating mechanisms. The August breeding individuals proposed above may be adaptively superior in the new environment and so August-breeding will become fixed and the two populations will be effectively isolated should they regain contact. Most isolating mechanisms, especially ethological ones, will not be adaptive in this way.

If the two populations diverge enough they may become genetically incompatible through chromosomal changes, and will be effectively isolated even though inter-mating may take place.

Most populations probably do not diverge to this extent before contact is re-established. If, on recontact, no niche-diversification has taken place, one species will eventually eliminate the other. If, on the other hand, both species are well adapted to their particular environments a certain amount of ecological isolation may intervene, but anyway, gene flow into such a population would be disadvantageous. Any members of the population that do not interbreed will be at an advantage because hybrids, although not necessarily infertile or inviable, will be adaptively inferior. They will be in the worst of both worlds and unable to compete successfully in either. In this situation, ethological or ecological barriers to interbreeding will become adaptively advantageous and will result in isolation.

It should be borne in mind however, that such mechanisms

cannot begin to evolve as a direct result of selection, until genetic incompatibility is sufficient to render hybrids sterile, or ill-adapted vehicles of 'gamete-wastage' - in other words, until speciation has been completed.

The most important parts of this scheme are that:-

a) Divergence between the two populations must be controlled by natural selection such that niche diversification has taken place.

b) The two populations must be so sufficiently well adapted to their own particular environments that hybrids are adaptively inferior.

c) Interbreeding can take place, at least initially, if the hybrids are adaptively inferior (or of course, inviable or sterile). Interbreeding does not necessarily imply gene flow.

d) Isolating mechanisms, having arisen at the zone of contact between the two populations, will eventually spread throughout the whole population if it is elsewhere adaptively neutral. It may even do so if it is inferior, if the disadvantages of the genes involved are less than the disadvantages of 'foreign' alleles being incorporated from the other population.

e) Further divergence, adaptation and speciation will follow until the two populations are genetically incompatible, in the sense that they are intersterile. Prior to this stage, although there may be no gene exchange between the populations, gamete wastage will occur.

'Good' species, isolated by the steps up to and involving c) can be found. The most famous example, quoted by Dobzhansky (1940); Mayr, (1942,1959b & 1963); Bigelow (1965); Key (1968) and others, is that of the two crows, Corvus corone (the carrion crow)

and C. cornix (the hooded crow) (Meise, 1928). The taxonomic status of these birds is open to question; Mayr (1959b) cites the two populations as examples of isolates that have 'not yet attained full species rank...' implying that they are sub-species as he suggested in 1963. However, he also refers to the two populations as separate species in both the 1959b and 1963 papers.

They occur in Europe, C. cornix in the north and east and corone in the south and west. There is a hybrid belt 75 - 100 km wide running from Scotland to the Mediterranean. The exact degree of fertility of the hybrids is not known but fertile back-crosses take place. Hybrids showing every conceivable combination of the parental characters and all degrees of intermediacy occur within the zone. Although the hybrid zone has been in existence since the last European glaciation, there is no evidence to suggest that it has broadened or that gene flow has reversed the divergence between the populations. "Selection seems to have very effectively weeded out those migrant genes that left their own well-integrated harmonious gene constellations to threaten the well-integrated (but different) harmony of the other." Bigelow (1965).

In this example, both populations seem to be good species, and interbreeding does not imply gene exchange between them. It is perhaps an unfortunate example to give of maintenance of isolation despite interbreeding, as no isolating mechanisms can be clearly detected at all. This leads to the question 'can populations attain and maintain species rank (separate gene pools) through the operation of natural selection alone?'.

This is obviously unlikely in most cases, spatial isolation

of one population from the other is usually necessary, but the field of isolation has probably aroused more controversy than any other aspect of speciation. It seems possible that the orthodox view of pre-speciation geographical isolation may not be correct in all cases of speciation.

c) Geographical isolation

The causes of intra-populational diversity, the material of speciation, have been summarised, as have the origins of isolating mechanisms whereby two populations can maintain their integrity after contact. It is now necessary to consider the circumstances during which species arise.

Two major, and quite distinct types of speciation have been proposed, allopatric and sympatric speciation.

Allopatric populations are defined by Mayr (1963) as "populations or species occupying mutually exclusive (but usually adjacent) geographical areas". Most species are allopatric in that they occupy different areas. One can disagree with Mayr's view however, in that most species are not adjacent.

Sympatry, defined by Mayr (1963), is the occurrence of two or more populations in the same area; more precisely, the existence of a population within the cruising range of individuals of another population.

A tentative classification of the elements of allopatric and sympatric speciation is given on the next page (138).

I. Allopatric speciation

i) isolation by geographical barriers

II. Sympatric speciation

ii) isolation by ecological barriers (including "allochronic" speciation)

iii) isolation by natural selection ("tension zones" and disruptive selection)

"Geographical isolation refers to the division of a gene pool in two by strictly extrinsic factors". It "is a physical separation of a portion of the species, permitting it to go its own way genetically." (Mayr, 1959). It is now generally accepted that geographical isolation is probably necessary for all speciation processes except polyploidy. "In general, geographic isolation seems to be a prerequisite for the development of genetic isolating mechanisms" (White 1959).

That geographical barriers are the major isolating factors in speciation has been stated by Lack (1945 & 1957); White, (1959 & 1968); Connel and Orias (1964); McArthur (1965) and by Mayr and Dobzhansky on many occasions.

The geographical barriers that give rise to this isolation may be obvious barriers such as stretches of sea, deserts and high mountains. Huxley (1942) gives an example of such barriers in operation where Salvelinus (char) have become confined to various lakes, fifteen different forms being distinguished in Great Britain and Northern Ireland. All of these are classified as sub-species, as 'once you begin giving specific names to lacustrine forms of char, you never know when to stop'. If all the char in G.B. & N.I. except a dozen were exterminated, each of the dozen would be recognised as a different species.

The speciation of Corvus corone and C. cornix occurred whilst the two populations were spatially separated by the glaciation of north western Europe, corone being confined to S.W. Europe and cornix to Eurasia.

Many examples of island fauna speciation during their isolation by stretches of sea are known. Most geographical barriers are not of this magnitude. In this context, White (1959) remarked that, "The very limited power of dispersal of many species of insects and other invertebrates has not been sufficiently taken into consideration", and cites the N. American grasshopper, Melanoplus in which the winged species are few and mostly occupy geographic areas of continental dimensions, even if they have rather specialised ecological requirements. The flightless species, on the other hand, are far more numerous and most of them are restricted to very small areas. The general conclusion is obvious, that a distance of a short flight by a grasshopper may represent an insurmountable barrier to a crawling insect.

"Ecological isolation" is also a form of geographical barrier, but it must be emphasised that the differing ecological preferences of two populations do not come under the heading of geographical isolation. Ecological isolation is merely micro-geographical isolation - similarly, geographical isolation is macro-ecological isolation. An oak woodland insect species with dispersal mechanisms operating over 1000metres will be geographically isolated if its woodlands are separated by a kilometre or so of pasture; whilst a widespread terrestrial species such as the rabbit in Europe is ecologically isolated from similar

habitats in north America by a stretch of unsuitable habitat - the Atlantic Ocean.

When postulating suitable geographical barriers in the evolution of a species, the power of dispersal is enormously important. Dispersal involves not only the obvious mechanics of movement, wings, seed dispersal mechanisms, &c., but also the ability to resist unfavourable environments during dispersal. The insect cited above may, during certain weather conditions, get blown to the next oakwood and be sufficiently fit on arrival to survive. The rabbit, on the other hand, is unlikely to survive the journey across the Atlantic without the intervention of man.

Geographical isolation has, as mentioned above, been largely accepted from Wagner (1841) and Darwin (1872) onwards as the prime, if not the only cause of isolation enabling speciation. There are cases however, where suitable barriers are difficult to postulate. Widely ranging species with good dispersal and unspecialised requirements will only be split into isolated populations by quite drastic barriers. Deserts, mountains and oceans, although representing formidable barriers to species such as the house sparrow, starling, rat and rabbit, do not arise very frequently. Much more difficult to understand is the speciation of such organisms as the Cetacea; most are sympatric and globally distributed and yet are distinct species with different food requirements. It is hard to postulate a geographical barrier that would have allowed the ancestral stock to speciate in this way. The same is true of many oceanic species of fish; although they may now have distinct food preferences and different times and places of breeding, orthodox views of speciation require prior

geographical isolation.

A different aspect of the problem is the speciation that occurs in strictly limited environments. Dobzhansky (1951) quotes the example of Darwin's finches (Geospiizinae) on the Galapagos Islands. That the island finches should diverge from those on the mainland is perhaps inevitable, but how fourteen different species have arisen in such a limited area is puzzling. Their power of dispersal must be good or they would not have got to the Galapagos in the first place.

It is even more difficult to envisage plausible geographical barriers to account for 'species-flocks' in lakes. Dobzhansky (1951) pointed out that there are 300 species of gammarids in Lake Baikal, and that this is more than the total number of species in the rest of the world. Fryer (1959) has investigated the fish fauna of Lake Nyasa and found over 180 species of Cichlidae alone, over 100 of which are in one genus. He pointed out that the lake is, at least at present, remarkably uniform in its physical characteristics, "there are few major habitats in the lake".

Sympatric speciation, where a new species arises within the range of the parental species, has never been widely accepted as has geographical speciation. For many years the theory seemed totally unacceptable. Mayr (1942) said of the theory of sympatric speciation, and not without reason, that it is "one of the most controversial subjects in the field of speciation. Unfortunately most of the discussions of this subject have been largely speculative and, if one attempts to gather well-substantiated data, one is surprised to find how little concrete knowledge

exists. The deplorable confusion of terms (is) not based either on adequate definitions or on an analysis of the concepts which they are supposed to support. This field is in such utter confusion (that as the) examples concern insects or marine invertebrates, and this makes a treatment of the subject even more difficult to an ornithologist...a better balanced treatment will have to be postponed...."

It is difficult to give a 'balanced treatment' of sympatric speciation as there is still 'deplorable confusion' of terms. Geographical isolation, or allopatric speciation, is a straightforward concept, covering a single mechanism that varies only by degrees. Sympatric speciation, on the other hand, is used to cover everthing else, whatever it may be, and includes all of those cases that will not be fitted into the scheme of allopatric speciation - whether or not there is another reasonable hypothesis to fit them.

The classification set out above (p. 138) is therefore not only tentative, but it is incomplete. A new concept of speciation described as sympatric arises whenever a particular group of organisms is investigated and will not fall into allopatric speciation. Such concepts are diverse and generalisations are difficult to draw.

Ecological speciation is the suggestion that 'biological' or ecological races of a species can coexist geographically in an area and gradually diverge genetically until they constitute species (White, 1968). Geographical races, each limited to its own slightly difference environment, are well known and frequently reported, but unless each race is confined to its own particular

habitat, separated from its nearest neighbouring race in its habitat, there can be no segregation of gene pools. If the races are separated by more than the cruising range of an individual, then they are microgeographically separated. There is no evidence to show that interspecific gaps can arise through habitat specialisation while each population is still in contact with another, and there is no known process that would allow the development of such gaps (Mayr, 1942).

There is however, one aspect of ecological separation that may allow speciation. Geographical isolation is isolation in the three spatial dimensions, and it seems possible that isolation in a fourth dimension, time, can allow of speciation. "Allochronic" speciation is the term coined by Alexander and Bigelow (1960) to refer to this process, which they postulate in the evolution of two species of field crickets (Acheta pennsylvanicus and A. veletis). Most of the evidence of distribution and ecology of these two species suggests that they have never been allopatric. They occur in N.E. North America, in climates that allow successful overwintering by eggs or late instar nymphs. As there is a consistent development rate, and only a short breeding season, the species mature and breed at different times, pennsylvanicus in Spring, and veletis in late Summer. Alexander and Bigelow (1960) suggest that the original population overwintered in all stages, with mature adults appearing and breeding throughout Spring and Summer. Because of a change in climate, or range extension into an area of harsher climate, only the two resistant stages were able to overwinter, thus effectively isolating the breeding populations. Ghent and Wallace (1958) have suggested similar allochronic speciation in sawflies, which can overwinter

as eggs or pupae. Gabbutt (1959) found that a European ground cricket, Nemobius sylvestris, has a two year life cycle, each individual overwintering as an egg the first year and as a nymph the next. The only possibility of gene exchange between populations mating in different years is by stragglers and these are not known to occur.

Alexander and Bigelow (1960) summarise the conditions needed to allow allochronic speciation as:-

i) a consistent developmental rate producing fewer than two generations per year,

ii) a narrow breeding season or short adult life,

iii) a duality in winter hardiness, with two stages involved, widely separated on the life history cycle.

In fact the third condition need not involve winter hardiness but any 'sieve' that allows through only individuals that are in phase, the phased populations being separated by a period of time greater than the breeding season.

Isolation by genetic barriers can only occur if a single genetic change produces reproductive isolation. Polyploidy is the obvious case, but this is unlikely to be of importance in sexually reproducing animals because of the disruption of the sex-determining mechanism, and has not been unequivocably demonstrated. Cases of polyploidy in animals are usually wrongly analysed cases of metacentric chromosome fission to give large numbers of acrocentric chromosomes (White, 1957).

Other genetic barriers have been postulated. White, Blackith, Blackith and Cheney (1967) proposed that the term

'stasipatric' speciation be used to describe isolation in species of grasshoppers by changes in chromosome number. Essentially, the process is seen as an expansion of the new chromosome type from its point of origin into the old population. Hybrids of old and new numbers are less viable or are sterile, and zones that thus arise between the populations act like semi-permeable membranes (Key, 1968), allowing some of the further genetic or chromosomal rearrangements to pass, but holding back others. Thus the new chromosomal type population is able to acquire adaptations without swamping, as most of the parental genes cannot pass the hybrid barrier.

"Two conclusions seem to follow from the facts. The first is that some types of chromosomal rearrangements function as fairly strong, primary genetic isolating mechanisms between incipient species of morabine grasshoppers. The narrowness of the zones of overlap is evidence of strong selection against fused chromosomes in 'unfused territory' and vice versa...." White (1968).

Key (1968) does not agree with White's interpretation of the situation. He proposed that the zones of hybridisation, the semi-permeable membranes, are zones of secondary intergradation, and that the races of differing chromosomal numbers have formed allopatrically, recontact being secondary.

Lewis (1969) referred to 'Speciation by chromosomal reorganisation with or without a change in basic chromosome number' as being prevalent in the plant genus Clarkia. However, unless large numbers of identical or chromosomal changes occur within the same population at the same time, all mutations and recombinations is sexually reproducing animals must pass through a certain

amount of heterozygosity before being fixed. If a new chromosome arrangement becomes heterozygote, it is no longer ensuring genetic isolation and under these circumstances speciation is impossible (White, 1957).

A third type of speciation was proposed by Huxley (1939) which may loosely be described as 'speciation by natural selection'. Where two large, relatively uniform, areas, occupied by a single species, are divided by a region of relatively rapid or unfavourable environmental change, two main gene types will arise. Both will be well adapted to their particular environments. While the population is continuous, these two gene types will interbreed. The hybrids between them are ex-hypothesis less well adapted and will be removed by natural selection, the two gene types remaining separate. Huxley (1939) proposed that the 'tension' in such a hybrid zone would lead to the speciation of the two halves, but Mayr (1942) objected:-

i) that continuous gene flow in the zone will prevent harmonious gene systems developing on either side to such a degree that there is hybrid inviability, or

ii) that a third balanced gene system will develop in the zone, guaranteeing unimpeded gene flow. On close examination, Mayr's objections would seem to cancel each other out.

More recently, Thoday and Boam (1959 & 1961) and Millicent and Thoday (1961) have shown that two populations of differing genotypes can be maintained by selection. Cultures of Drosophila melanogaster were set up with various mating systems, some of which allowed 50% of the genes in one generation to be derived from the other population. This is twice the gene flow that would be involved in random mating and obviously more than that involved

if a certain amount of selective intra-population mating were to take place. Individuals were selected on the basis of sterno-pleural chaetae number, those with high and low numbers were used as breeding stock for the next generation (allowing a given quantity of gene flow) whilst those with median numbers were discarded.

Their conclusions were that no mating system up to 50% gene flow could prevent divergence and that 'isolation is not a prerequisite of divergence under divergent selection pressures'.

These experiments did not show the initiation of isolating mechanisms but they were only continued for a relatively short time (10 generations in the case of the random mating system). However, chromosomal differences between the two populations did arise and under these circumstances it seems likely that selection pressure could produce isolating mechanisms in wild populations.

Fryer (1959) has proposed that the species of cichlid fishes in Lake Nyasa have arisen in large numbers because of disruptive selection by predators. There are few different types of habitat around the shores of L. Nyasa and each is reproduced many times, separated from similar habitats by stretches of different habitats. "It has been emphasised how, in the littoral zone, a given species of fish usually is rigorously restricted to a given habitat. Now, if for any reason, a fish from say a rocky shore should attempt to cross a strip of sand or <u>vice versa</u> it would, as the result of its colouration and ill-adapted escape reaction in that habitat, be particularly easy prey for a predatory fish". Fryer envisages an original sandy shore

population, inhabiting discontinuous habitats (although within the cruising range of the next patch) being isolated by the extreme pressure of predation. Evolution and speciation thus continues along independent lines on each piece of sandy shore.

To summarise, geographical isolation of some degree is the accepted, and probably usual, method of speciation. Polyploidy, at least in plants, is the only other established mode of speciation, but it is at least possible that allochronic speciation can occur in certain types of animals. Disruptive natural selection may play an unsuspectedly large part in the speciation of many other types.

d) Rates of speciation

Estimates of the rate of speciation, that is, the time necessary for a population to acquire specific differences after initial isolation, can only be arrived at circumstantially. "Speciation is a process which probably always takes more than a hundred thousand years, and may usually take more than a million years. The process as a whole is hence not open to investigation by experimental methods" (White, 1959).

The time necessary for speciation will depend upon the degree of difference needed between two populations to give specific distinction and the rate of change in those populations. Rates of populational change are themselves a function of the rate of genetic change (mutations and recombinations of genes and chromosomes) and the effects of natural selection upon these changes.

The taxonomic characters used to distinguish 'good' closely related species may depend on as few as 20 gene substitutions, but non-detectable substitutions will also have taken place (Haldane, 1957). Haldane believes that 'good' vertebrate species, even when closely related, may differ at several thousand loci, out of a total per species of about 40,000. Speciation rates will not be affected if some of the genes control only slight differences.

The rate of mutation of any one gene seems to be between once in 10^4 to once in 10^7 zygotes (Dobzhansky, 1951; Haldane, 1957; Mayr, 1963). However, most mutation rates have been estimated using lethal or 'drastic' genes, and Mayr (1963) suspects that mild genes, which probably contribute most to genetic variation, have frequencies three or four times as high. White (1945) suggested that the mutation rate in any group would be affected by the length of the X-chromosome. The assumption is that the larger X-chromosomes carry more genes, which are sex-linked, than do smaller X-chromosomes; and these sex-linked genes are as exposed to natural selection as are dominants or homozygous recessives. They will thus be eliminated quickly, if deleterious, and natural selection will tend to select against mutations that increase the general mutation rate.

Rates of chromosome mutation and recombination are difficult to estimate because the effects produced are usually phenotypically the same as genetic effects, and the rates given above may include chromosome changes as well as gene mutations.

Haldane (1954) estimated rates of natural selection in wild populations as being between zero and 12%. This estimate is the

differential mortality between the 'best' phenotype (i.e. the one with the lowest mortality) and the average mortality of the population. Intense selection of 12% means that 12% more individuals of the optimum phenotype survive than the average of the population. What effect the intensity of natural selection has on speciation rates is difficult to gauge, but Haldane (1957) believes that the cost of gene substitution by natural selection is usually much the same unless selection is very intense.

The materials of evolution, that is genetic change, and natural selection interact to produce rates of genetic change within populations. "The principal unit process in evolution is the substitution of one gene for another at the same locus. The substitution of a new gene order, a duplication, a deficiency, and so on, is a formally similar process" (Haldane, 1957). Natural selection acting on genotypes will eventually result in changes in gene frequency, and it is this process that produces specific differences according to Haldane. He calculated that "the substitution of one allele by another.....usually involves a number of deaths equal to about 10 or 20 times the number in a generation....and perhaps rarely being 100 times this number". In evolutionary terms, this means that the mean rate of gene substitution will be one per 300 generations. It may be much longer if allele a_1 is replaced by allele a_{10}, the population passing through stages of fixation of a_2, a_3, &c.

Haldane (1957) doubts that this rather slow rate of gene substitution can be speeded up by selecting several genes at once. With novel genes occurring at a frequency of 10^{-4} (the average mutation rate) only one individual in 10^{12} will possess

three given mutations at once, and the cost of selecting its descendents would be the same as that of selection for the three mutations in series.

Evolution may be further slowed by the necessity of substituting genes in series.

Estimates for actual speciation very somewhat. Haldane using the calculations outlined above, and a specific difference of 1000 loci, suggested that at least 300,000 generations are needed for specific differences to emerge. Other estimates are usually given in terms of years and they may, of course, represent very different numbers of generations depending on the organism.

Mayr (1963) pointed out that the rate of speciation may vary considerably; some isolated marine faunas have not generated specific differences in more than 3,000,000 years, whilst some lake 'species flocks' of fish have arisen in 100,000 years. This last figure agrees with that of White (1959), whilst Zeuner (1945) using data from the Pleistocene fossil record, concluded that in mammals, about 500,000 years were required for the evolution of a new species. Beirne (1947), quoted by Simpson (1953), also working on Pleistocene and post-Pleistocene data, found that 57% of modern British land mammals have evolved specific differences since the end of the last interglacial. This was estimated by Zeuner (1945) as 80,000 years BP (before present), but modern authors have disputed this figure, and Cornwall (1970) dates the end of the last interglacial (the beginning of the Würm glaciation) at 115,000 years BP. Beirne (1947) found that no specific differences had evolved since the Würm glaciation, Extensions 2 - 3 (72,000 BP to present - Cornwall, 1970).

Sub-specific differences are not as meaningful, as the actual degree of difference involved can vary considerably; but Mayr (1942) gave a figure of 5,000 to 15,000 years for the evolution of such differences since the Ice Age. Simpson (1953) drew attention to the seal, Phoca vitulina, one form of which has been isolated in a Canadian lake for 3,000 - 8,000 years and is now accorded sub-specific rank. Obviously actual rates of speciation vary. The brachiopod, Lingula, has remained taxonomically unchanged for 400 million years, whilst the mouse, Apodemus sylvaticus, has reached sub-specific status in the 1,000 years of its isolation on Iceland (Simpson, 1953).

Most authors are agreed, however, that speciation in vertebrates never takes less than 100,000 years. Upper limits, as can be seen from the case of Lingula, are rather meaningless, although Zeuner (1945) gave an absolute maximum of 500,000 years for species steps in mammals.

The rate of evolution will depend on the environment, its heterogeneity, and its absence of predators and/or competitors of the incipient species; and the genetic make up of the isolate. Marine species with large gene pools will usually evolve and therefore speciate, slowly; whilst terrestrial organisms with short generation times and small populations will speciate relatively quickly.

5 Lampreys and orthodox speciation theories

The orthodox and most usual process of speciation may be summarised as follows:-

1) An originally continuous population with inherent genetic and chromosomal variability, becomes divided by some geographical barrier.

2) Each part of the population will evolve independently as a result of i) initial differences in gene frequency

ii) mutations and recombinations being asynchronous in each population

iii) adaptation to somewhat different environments.

3) If the populations remain spatially isolated they may gain genetic isolation purely through divergence during isolation.

4) If the populations regain contact and divergence during isolation has been sufficient, isolating mechanisms will arise or existing ones strengthened, and the populations will become 'good' species.

5) If sufficient niche diversification has taken place, they will continue to coexist.

6) The process of genetic change, sufficient to generate species differences will probably take between 100,000 and 300,000 generations.

Given this framework and the implied constraints, it is now necessary to examine the taxonomic status of L. fluviatilis and L. planeri.

They are classified as different species but the speciation of planeri from fluviatilis cannot be reconciled with orthodox view of speciation.

The major features in speciation are intrapopulational genotypic variation, the origin and fixation of isolating mechanisms and isolation.

It must be assumed that fluviatilis has some degree of genetic variation. Individuals differ in size and colouration, but much of this variation may be the result of environmental effects. However, no natural population has been found that does not show intrinsic variation and the mechanisms of meiosis and mutation make it unlikely that such a population could exist.

It is possible that the rates of change due to structural chromosomal alterations, translocations, substitutions, deletions, &c., may be fairly high in lampreys because of an unusually high chromosome number. Figures for each species vary, but most species have chromosome complements within the range 120 to 170 (Chubareva, 1957; Lanzing, 1959; Nogusa, 1960; Zanandrea and Capanna, 1964; Sasaki and Hitotsumachi, 1967; Howell and Denton, 1968 and Potter and Rothwell, 1970).

If such chromosome changes do increase the intrinsic population variation, it is possible that this is counteracted by selection against genes increasing the general mutation rate, as the load of variation within the population would tend to make too many individuals ill adapted.

It has been suggested that high chromosome numbers are the result of polyploidy, but although no sex chromosomes have been identified in lampreys, it is probable that polyploidy would drastically interfere with the sex determination mechanism. As

lamprey chromosomes are probably all acrocentric the high numbers are probably the result of metacentric fission and occasional chromosome duplication.

Assuming that the basic variation necessary for evolution and speciation is present in lamprey populations, what isolating mechanisms have arisen between fluviatilis and planeri? Both lampreys are sympatric during their ammocoete life, but although this may raise questions in relation to Gause's principle and competition it is not relevant from the point of view of isolating mechanisms. Only that time during which both planeri and fluviatilis are in breeding condition is important.

Of the possible isolating mechanisms, geographical isolation, as discussed above, is not a true isolating mechanism in that it is not intrinsic to the population. It will, of course, serve to prevent gene exchange between species and this is relevant.

From the data given in the section on Distribution (p. 9 et seq.) and from our own field observations, it is probable that fluviatilis is nowhere present in river systems that do not also contain planeri. L. planeri are found, however, in streams in which fluviatilis adults or ammocoetes have not been found. Their global distribution is identical as would be expected if planeri has been derived from fluviatilis.

Habitat isolation during spawning usually occurs as planeri spawn in small streams, or higher up river than fluviatilis. However, planeri, showing redd building behaviour, have been found in fluviatilis redds on all of the fluviatilis spawning grounds studied. Although planeri can use fluviatilis redds, it is not likely that fluviatilis could build or use redds in the small gravel sections of streams usually chosen by planeri.

Temporal isolation is difficult to assess. Most of the planeri redds closely studied have been in streams in which fluviatilis do not occur, but the evidence suggests that both planeri and fluviatilis begin redd building when the water temperature reaches 11°C. Fluviatilis may be slightly earlier than planeri but, as mentioned above, planeri have been found in fluviatilis redds while they were occupied by the spawning river lampreys.

Pre-fertilisation sexual isolation is brought about by ethological discrimination or by non-correspondence in size of the adults. The patterns of spawning behaviour seem to be the same in both planeri and fluviatilis, although the latter has not been studied so closely. However, male planeri have been seen attempting to pair with female fluviatilis (Huggins and Thompson, 1970 and personal observation). It is impossible to judge the success of these attempts because the shedding of eggs or milt has never been observed, even in aquarium observations.

Size discrepancies make the normal coupling position impossible between individuals of planeri and fluviatilis. This may not be important if the coupling behaviour is sufficiently successful to induce the release of gametes as fertilisation is external. Although Kille (1960) reported a sperm-mobility life of 50 sec we have found that fertilisation can occur after considerably longer delays than this (4min).

Post-fertilisation isolation mechanisms are based on the viability or sterility of gametes or hybrids. There seems to be no gametic isolation between the two animals because artificial fertilisation is as successful between fluv/plan crosses as it

is in the homologous crosses. Hybrids seem as viable as homologous crosses up to two years after fertilisation. However, there is a very high mortality in all crosses in aquarium populations and this may mask some hybrid inviability. Whether hybrids would survive metamorphosis and what their subsequent development and behaviour would be is not known because the present aquarium populations are only 2 years old. For this reason also, there is no evidence on hybrid sterility or hybrid breakdown (inviability or sterility of the F_2 generation).

Isolating mechanisms between planeri and fluviatilis may be summarised as follows:-

1) Partial geographical isolation of planeri from fluviatilis.
2) Partial habitat isolation of planeri from fluviatilis.
3) Possible sexual isolation on the basis of size.
4) Possible hybrid inviability in later ammocoete life.
5) Hybrid sterility - no information.

No isolating mechanisms are known therefore, that would ensure complete segregation of the gene pools. As planeri and fluviatilis have been found spawning together and it is known that hybrids are initially viable, it is likely that hybrids do occur, at least occasionally, in the wild. As planeri can spawn in fluviatilis redds, but almost certainly fluviatilis cannot spawn in planeri redds, gene flow will tend to be from planeri to fluviatilis, unless hybrids are sufficiently viable and fertile to return planeri genes with an admixture of fluviatilis genes in a later generation.

The process of speciation usually occurs during and after geographical isolation. The barriers likely to be experienced

by fluviatilis may be classified as those that prevent gene exchange between parts of the population within a river system and those that prevent gene exchange between populations in different river systems.

The first type are all barriers to migration. The flow of the river is stopped, either along its course, in which case populations in the upper reaches are 'landlocked'; or at the mouth, in which case the population of the whole river is landlocked. The phenomena that would present this type of barrier are as follows:-

1) Sand banks or gravel shoals across the mouth of the river. A pebble bank moving inshore, such as Chesil Beach and that at Slapton Sands, will block the mouth of a river and prevent migration, but still allow a certain amount of current to flow. Sandbanks are less liable to block the mouth of the river as they are easily eroded and do not allow the ready passage of river water.

2) Waterfalls.

3) Glacial moraines, landslides or other debris across the river.

4) Glaciers.

5) Man-made barriers of which dams and weirs are obvious examples, but stretches of intensely polluted river may also bar migrants.

The advantage of postulating this type of barrier in the derivation of planeri is that a non-parasitic, non-migrating type of lamprey would be the obvious adaptive response of a landlocked fluviatilis population.

However, certain features of these barriers make it unlikely that they are the prime cause of speciation.

Debris barriers, sandbanks, landslides, moraines, &c are temporary. A lake formed behind them will rapidly overflow and overflow channels of this type are quickly deepened and the barrier eroded.

Waterfalls, on the other hand, are more permanent, although temporary on a geological time scale. However, they will only prevent upstream migration and lampreys such as fluviatilis with an 'inbuilt' downstream migration will merely disappear from the reaches above the waterfall.

Glaciers will block a river more effectively and for longer than debris barriers. Overflow channels will arise but they will be cut through the hard surrounding rock or constantly replaced ice rather than through soft, unconsolidated material. For this reason the gradients will remain steep and may constitute a successful barrier to migration.

All of these barriers, except waterfalls, will arise quickly and would seem to call for instant adaptation. Certainly most of them are so ephemeral that instant adaptation is necessary to prevent swamping by fluviatilis types on their early recontact. If fluviatilis is in fact an estuarine feeder, it will be by nature tolerant of low salinities and such an immediate change in life history may be possible.

This change would be the result of individual adaptability rather than adaptation of the population as the impermanence of the barriers would not allow time for the latter. As Gause (1942) pointed out, there is usually an inverse relationship between adaptability and adaptation, that is, the better the original

adaptability, the less likely is the population to undergo adaptation.

In this particular case, if the fluviatilis type is able to withstand such a sudden change of environment as landlocking would produce, it is because conditions fall within the ranges of tolerance of at least some of the population. Unless migration is prevented for sufficiently long to allow of considerable genetic change, a return to a fluviatilis type of life history will occur on the disappearance of the barrier.

In most of Europe, any freshwater populations must have arisen since the Ice Age. There is general agreement that the length of the post-glacial period has been insufficient to allow much genetic change, even in one-generation-per-year organisms.

The second type of isolation, the restriction of populations to single river systems would be the inevitable result of 'inbuilt' 'homing' behaviour, such that each lamprey returned to the river or river system in which it spent its ammocoete life. This type of isolation is continuous and would allow sufficient time for the normal processes of genetic change and adaptation.

However, homing has never been shown in lampreys and the difficulty of marking downstream migrants for subsequent recapture on their upstream migration suggests that it is a difficult subject to investigate. The homing mechanism would have to be strongly imprinted as only occasional 'off-course' stragglers would allow gene flow. Although salmon are known to home, they have not evolved into single-river-system races, presumably because stragglers are fairly common.

If homing behaviour is sufficiently imprinted to maintain

genetic isolation is presumed, it is difficult to account for the initial spread of fluviatilis into previously uncolonised areas to give their present day distribution. Also on this basis, it must be postulated that all rivers containing both planeri and fluviatilis have been recolonised by planeri.

The production of non-parasitic, non-migratory planeri is not an adaptive response to isolation within a river system and if this mechanism is postulated, it is necessary to investigate the causes of the production of a planeri-type. This is made more difficult since it implies that the total populations of certain river systems must have evolved into planeri-types. There are no downstream barriers here, only barriers between river systems and so the gene flow within a river will be maintained. It is unlikely that selection pressures could be such that all the fluviatilis of one river system evolve into planeri, whilst none of these do within another river.

Without planeri dispersal a landlocking origin would only produce planeri in rivers that have a fluviatilis population whilst within-river isolation would never produce streams with both planeri and fluviatilis.

There is no evidence that planeri do spread from their point of origin but freshwater fishes colonise new stretches of water and presumably the mechanisms for both these fishes and planeri are much the same.

Kingfishers are known to take adult planeri. Although kingfishers are normally restricted to short stretches of a single stream, there is the possibility that an occasional adult planeri may be dropped where it can find its way into another water course.

Herons are more likely to be the chief dispersal agent. They have been seen on the spawning grounds of planeri of the river Yeo (N. Somerset) while the lampreys were spawning, and heron footprints are often found on ammocoete mudbanks. As eels are frequently taken by these birds it is likely that they occasionally catch adults or ammocoetes of planeri. Lampreys may be dropped or regurgitated alive if the heron is alarmed, or they may fall through the nest during the feeding of young herons. As heronries are often built by the waterside and as individual herons fish several streams within an area, there is the possibility of colonisation by this means. (Lowe, 1954, described the life of herons).

More probable however, is the transfer of eggs on the feet of wading birds such as herons, especially as the eggs are sticky for a short time after fertilisation. Moorhens, coots and small waders such as the sandpiper may also be involved.

Dispersal by these methods is likely to be slow, and in steps of up to a mile. Sufficient eggs or individuals must be transferred at the same time to the same place to give a breeding population and until the new population has been built up, the next step in dispersal cannot take place.

Neither landlocking nor origin with isolated river systems are very satisfactory as the cause of the isolation necessary for orthodox speciation. With the probably rather limited dispersal of planeri, too many barriers of a type that is both infrequent and impermanent would be required for landlocking. Isolation within a river system would involve an unlikely degree of homing, an evolutionary response that seems unlikely to be advantageous under the circumstances and a rather high degree of dispersal of planeri.

As mentioned above, Haldane (1957) believes that 300,000 generations are necessary in vertebrate evolution to produce a specific difference. Taking one lamprey generation as seven years, and there is no reason to suppose that it has recently lengthened, the origin of planeri would be just over 2,000,000yrs BP. If planeri differs from fluviatilis by only half of the number of genes that Haldane suggests is necessary for a specific difference in the vertebrates and his lowest estimate for the cost of substitution of a single gene is taken, the origin of planeri could be as recent as 350,000 years

However, all recent estimates of the timing of Pleistocene events agree that the last glaciation (Würm) began 100,000 to 200,000 years ago and did not end until between 10,000 and 15,000 yrs BP (Cornwall, 1970; Ericson, Ewing and Wollin, 1964; and Godwin, 1961). The latter part of this period can be investigated by radio-carbon dating.

According to different estimates, planeri can therefore have arisen at any time between the Tertiary period and the middle of the last glaciation. It seems unlikely however, that a solely freshwater species such as planeri could have survived such glacial and periglacial conditions as were prevalent over most of its range during the Ice Age.

If its origin were pre-glacial, reservoir populations could have survived in parts of the Mediterranean peninsulas and Iberia. To subsequently spread through Europe on the retreat of the glaciers, the Alps or Pyrenees must have been crossed, and the probable dispersal agents are unlikely to have been effective in these regions.

To colonise Britain, planeri must have reached northern France by about 10,000 yrs BP when eustatic sea level rises flooded the English Channel. The vegetation at this time was still tundra (Sissons, 1964). It seems improbable, therefore, that planeri would spread so rapidly through areas where the climate was harsher than in its present range, or it would surely have a larger range than at present, and where its probable principal agents of dispersal were absent.

If the speciation of planeri has taken even a quarter of the time that is estimated for other European Pleistocene fauna, it must either be very resistant to freezing, or have dispersal mechanisms that are better than those of many land mammals and as good as those of many birds.

From the foregoing discussion, it can be seen that:-

1) There are no known isolating mechanisms that would serve to completely isolate fluviatilis and planeri genetically.

2) Geographical barriers allowing orthodox speciation are difficult to postulate.

3) The time taken for the speciation of planeri must be of a much lower order of magnitude than that proposed for vertebrate species. Invertebrate speciation is even slower than that of the vertebrates.

Alternatively, planeri possesses unlikely powers of resistance or dispersal.

As the orthodox speciation concepts appear inadequate when applied to planeri and fluviatilis, as they do occasionally to other groups of organisms, two conclusions can be reached:-

1) there has been a different mode of speciation involved in the origin of planeri, or,

2) planeri and fluviatilis have not speciated, i.e. they are not good species.

6 Models of speciation of lampreys

Whether L. planeri and L. fluviatilis are good species or not may well be a matter of definition. If it is accepted that planeri has arisen in situ over most of its range since the Pleistocene however, the time available for differentiation from fluviatilis seems to be inadequate to allow for true speciation.

Whatever taxonomic label is applied for convenience to the degree of difference between planeri and fluviatilis, the differences themselves are real, and any theory of the origin of planeri must explain these differences as far as is possible.

The differences between fluviatilis and planeri are probably mainly the result of a delayed metamorphosis of planeri; the larger size at metamorphosis being an obvious example. Smaller adult size in planeri will follow delayed metamorphosis because filter-feeding is an inefficient mechanism, expecially with a large body size; and it is to be expected that the two extra years of larval life do not fully compensate for the loss of the 12 - 18 months parasitic feeding phase of the adult fluviatilis. If, as seems likely, egg production is related to size, the lowered fecundity of planeri and other brook lamprey types is also a result of delayed metamorphosis.

A largely notional type of fluviatilis, the 'praecox' form, has been proposed from time to time. It is not clear what is

mean/by the *praecox* form, only that it is a smaller type of *fluviatilis*, possibly with a middle-length larval life of 5½ years, and/or a freshwater feeding adult phase. A theory of origin of *planeri* would preferably accommodate the existence of this *praecox* type, whether or not it actually occurs.

The theory of the origin of *planeri* set out below is unsatisfactory in many respects. It is felt, however, that orthodox speciation, involving geographical isolation and the development of isolating mechanisms on recontact is just as unlikely, and that the data available point to a rather different mode of origin for *planeri*.

The argument below is set out as a series of statements, although many of the stages are hypothetical. Where a given hypothesis is important throughout, or at two different stages, it may be repeated.

1) A river, above certain minimum size supports a population of *fluviatilis* that has recolonised it since the last glaciation.

2) The river has smaller stones in its tributaries and headwaters than further down the mainstream. (While this conflicts with accepted hydrographical theories, it is in our experience, true for the stretches of river in which spawning lampreys are found. Although the mountain and upland tributaries tend to have large boulders, and the lowlands have mud and silt, most middle reaches appear to have stone sizes graded as postulated.)

3) The *fluviatilis* population has a certain amount of inherent variation, some of it genotypic, and some merely the result of environmental differences.

4) Some of this variation is in adult size, and some of this is genetically controlled.

5) A lamprey of a given adult size can only build a redd between certain, quite well defined limits because:-

a) If the stones of the river bed are too large, the lamprey cannot move them.

b) If the diameters of the stones of the river bed are much smaller than the diameter of the buccal disc, the lamprey cannot pick them up and if the stones are too small, they will not serve to anchor the female during pairing.

6) During the pre-spawning migration, the adults will move upstream until stopped by a barrier, e.g. a waterfall, or until they reach the limit imposed by stone size (5b). There will be a strong selective pressure to give the animals an instinctive drive to migrate as far upstream as possible.

7) Smaller adults will be able to move slightly further upstream than large adults until limited by stone size and this will tend to produce sorting along the river by size of the animals.

8) Where size is genotypic, this sorting will be increasingly reinforced in subsequent generations; i.e. genotypically small lampreys are more likely to breed with other small genotypes than with large genotypes, and this genotypic trend will be progressive with time until some other limit is found (see below).

9) When this sorting has been maintained for a few generations the population will tend to become a series of ecological races. Matings will tend to take place in intra-size-groups rather than inter-size-groups, and the gradation will be reinforced. There is however, no genetic discontinuity

between groups. The largest and the smallest length groups will be joined by all of the intermediate gradations, through which there will be gene flow, although this will be reduced.

10) Both upstream and downstream there are ecological limits to the range of expansion of the fluviatilis population, even if the small ones continue getting smaller and can use smaller stones and the larger increase in size and can use larger stones.

a) The large sized animals downstream are probably more fecund because of their larger size, than those upstream. Downstream 'flushing' of the ammocoetes and the consequent mortality is high however. An ammocoete settling in a mud-bank well downstream is much more likely to get swept to sea by flood water before maturity than is an upstream ammocoete. Larval mortality in downstream populations may also be heightened by the limited number of suitable mudbanks available between the spawning site and the tidal brackish reaches. The downstream limit of the population therefore, is reached when the high fecundity can no longer compensate for the high larval mortality. P. marinus, which is larger and spawns further downstream, has a much greater fecundity. This is probably supported by a much longer marine feeding stage than that in fluviatilis.

b) Upstream limits of the population will be reached when the relatively low fecundity of small fluviatilis cannot compensate for mortality. Larval mortality in these upstream spawning populations will be much reduced because the ammocoetes can use many more suitable mudbanks than can the down-

stream populations, much of the river being available to them. The likelihood of upstream ammocoetes being flushed out to sea will also be much reduced. However, migration mortality will be much greater. During the life of the ammocoete there is a tendency for it to be carried downstream from mudbank to mudbank, but the journey to the sea and back during its adult life will be longer and consequently the duration of the adult feeding period may be less.

The upper limit of the expansion of the population in the river may therefore be reached when the food reserves built up during the marine feeding phase are insufficient to support the animal during a long upstream migration and its subsequent spawning - bearing in mind its reduced fecundity.

11) Gene flow between the ends of the population will be reduced by distance and in comparison with many other animals it will be slow because of the relatively long generation time.

12) The population of small-sized animals will pass genes to the larger sized animals more easily than vice versa, because the small lampreys can use large redds once they are built, but the larger animals cannot use small redds.

13) The large fluviatilis are committed to migration to sustain the fecundity necessary to counter the high larval mortality. Genes for an abbreviated migration or shorter adult life may arise but they will be severely selected against.

14) There is severe selection against the small-sized populations becoming even smaller as they could not then complete adult life and migration successfully (10b). There are advantages

in smallness however, as the larvae can exploit more of the river.

15) Any change which will curtail migration in the small populations will remove the selective disadvantage of migration and allow the full exploitation of the advantages of small size. With a non-migrant small sized adult, these advantages would be:-

a) reduced mortality due to flushing because the ammocoetes are well upstream to begin with.

b) increased numbers of mudbanks for exploitation down the length of the river.

c) new spawning sites are available upstream. If the original small-sized animals' upstream limits had been set by migration difficulties rather than stone size, the removal of migration will allow expansion upstream.

16) The possible patterns of non-migrating life history are:-

a) the curtailment of adult life, i.e. 4½ years larval life with spawning following metamorphosis at 5 years.

b) neoteny, i.e. precocious sexual development at some time before metamorphosis. Spawning would occur at up to 4½ years.

c) delayed metamorphosis, i.e. lengthened larval life to 6½ years with spawning following metamorphosis, at 7 years.

a) and b) above will produce a very small adult and, if has been suggested, egg production is limited by adult size, the fecundity of these types will be very low. Scheme b) has the further disadvantage of giving no adult form. It is unlikely that a larval form would be as efficient at upstream swimming as the adult, and of course, redd building and spawning

on the usual lamprey pattern would be impossible, as would be the persistence of the population therefore.
Scheme c) provides an adult form to enable redd building and spawning. Adult size will be larger than those provided by a) or b), as there is a compensatory period of larval feeding life but the adult would not be as big as one that had a parasitic trophic phase.

If a), b) and c) are all under the control of equally simple timing mechanisms, c) will still be the most advantageous type of change. The mortality from migration upstream is eliminated and the adult phase necessary for redd building and spawning is retained. While the adult is reduced in size sufficiently to exploit smaller-stoned upstream reaches and thus further reduce larval mortality by flushing, it is not so small as to reduce the fecundity to unacceptably low limits. Delayed metamorphosis would therefore be the most advantageous adaptation for small, high-river fluviatilis.

17) As both metamorphosis and breeding occur during short periods at a particular time of the year, the delay in metamorphosis would be saltatory, i.e. in one year jumps.

18) Despite the delay of metamorphosis in part of the original population, there will not be genetic isolation. The smaller size of the 'brook-lamprey' type will tend to isolate individuals upstream and therefore further reduce flow, but as remarked above, some gene flow from the small-sized population to the larger sized is likely to continue.

It is possible that planeri and fluviatilis have reached this degree of separation and progressed no further to date. It has

been reported above that artificially produced hybrids are possible and that planeri have been seen spawning in fluviatilis redds. If natural hybrids are possible, there will almost certainly be gene flow between the populations, which will not, therefore, be good species.

As the conditions outlined above are continuing at present, it can be expected that planeri and fluviatilis will remain distinct forms. Segregation by size continues and will tend to counteract gene flow. Further steps in the speciation of planeri from fluviatilis would be the development of reproductively isolating mechanisms. Such steps may already have taken place, but as mentioned in p. 155 et seq.above, reproductive isolation does not yet seem to be complete.

The steps listed above outline the selective forces that have made the development of delayed metamorphosis in a part of the fluviatilis population advantageous. They have not proposed a genetic mechanism for delayed metamorphosis and the following theories attempt to offer this.

I. Transition through praecox.

1) The timing of metamorphosis is controlled by genes at a single locus.

2) The normal fluviatilis type, with 4½ years of ammocoete life and 2½ years as a migrating and marine feeding adult, has a pair of genes AA, 'setting' metamorphosis at 4½ years.

3) Mutation rates of lethals and 'drastics' are about 1 in 10,000. A population of 100,000 adult fluviatilis would be expected to produce, on average, 20 adults per generation with a mutant genotype Aa.

4) If the mutant 'a' has a modifying effect on 'A' such that metamorphosis is delayed for one year, the individuals with the Aa genotype will have a larval life of 5½ years.

5) Such mutants will have a reduced adult life of 1½ years and are called here, for convenience, praecox.

6) On metamorphosis, praecox will migrate. If they are produced in a short river system, the time necessary for migration may be sufficiently short to allow an adequate marine feeding phase.

7) Alternatively, if fluviatilis and praecox are tolerant of low salinities as adults, as fluviatilis must be in its late adult phase during upstream migration, praecox may be able to feed in estuaries or even fresh water, given a suitable fish population.

8) Praecox adults will be smaller than normal fluviatilis and returning up river will tend to spawn higher.

9) The relative fecundities of planeri (ca. 1,500 eggs), fluviatilis (ca. 20,000 eggs) and marinus (ca. 170,000 eggs) suggest that even a short upstream displacement of the population greatly reduces mortality.

10) Offspring of praecox x praecox crosses will have metamorphosis timing genes AA, Aa or aa. The offspring will be in the proportions of 1 fluviatilis: 2 praecox: 1 planeri.

11) AA types are normal fluviatilis and eventually return to the parental fluviatilis population. The praecox may or may not persist depending upon prevailing conditions.

12) aa types have a double 'dose' of delaying genes and the time of metamorphosis is delayed a further year, i.e. to 6½ years.

13) The twenty original adult *praecox* would be expected to produce 5 adult aa-*planeri* types, if mortality were the same as that in *fluviatilis*.

14) Migration mortality of the aa offspring of *praecox*-*praecox* crosses is eliminated, and the larval mortality is reduced by a slightly higher upstream spawning.

15) Mortality, as expressed by egg numbers, is fifteen times as great in *fluviatilis* as it is in *planeri*. Given that the new '*planeri*'experience a much lower mortality, 20 adult *praecox* may be expected to produce perhaps, 30 or more *planeri* type adults.

16) These *planeri* adults would breed truly, being aa genotypes.

17) Second generation *planeri* will accrue the full advantage of spawning higher upstream.

18) Further *praecox* types will be produded each generation of *fluviatilis* by mutation and they in turn will produce *planeri*.

19) Crossing of *praecox* and *fluviatilis* will produce *fluviatilis* and *praecox* offspring in equal proportions; crossing of *praecox* and *planeri* will produce equal proportions of *planeri* and *praecox*.

II. Direct transition from *fluviatilis* to *planeri*.

1) The timing of metamorphosis in *fluviatilis* is controlled by genes at a single locus.

2) The normal genotype is AA, which produces a metamorphosis at 4½ years.

3) Part of the population carries a gene 'a' which has arisen by mutation.

4) Gene A is either dominant to a, or if equal in effect, appears dominant, as its effect is chronologically earlier. The metamorphosis cannot occur twice.

5) The homozygote 'aa' delays metamorphosis by two years.

6) 'a' is neutral in the heterozygous state because of the effective dominance of 'A'. It will tend therefore to spread randomly throughout the population, its frequency increasing by further mutations until its mutation rate is balanced by the rate of back mutation from 'a' to 'A'. If forward and backward mutation rates are equal an equilibrium will be established when there are equal numbers of the two alleles.

7) When the population carries equal numbers of genes a and A, the offspring of each generation will be in the proportions of 1AA:2Aa:1aa.

8) aa's, whenever occurring,metamorphose two years later than the AA and Aa fluviatilis to become planeri.

9) Planeri (aa) arising in down river parts of the population will be eliminated as lowered fecundity will only be offset by non-migration and not by a lowered larval mortality. Those arising in the higher, smaller-fluviatilis populations however, will have an already somewhat decreased larval mortality, and may be expected to survive.

10) Planeri x fluviatilis crosses would give Aa or aa offspring, (fluviatilis and planeri)

The disadvantages of Scheme I.

A weakness of this theory of origin of planeri is that the initial fluviatilis populations must be fairly large - probably with 10,000 or more breeding adults. There are no estimates of fluviatilis population sizes, but from our own experience of the

Tewkesbury weir trap on the Severn river, populations several times this size can be found in some river systems. There are many historical records of large numbers of fluviatilis caught as food, many towns filling a yearly quota to send to Court. It seems probable that fishing and pollution have reduced populations of both anadromous lampreys in historical times but populations of fluviatilis of 10,000 or more have probably occurred in many rivers at some time since the Ice Age.

If praecox arise in any one generation from a fluviatilis population, they must survive to breed if they are to give rise to planeri. In most rivers, at most times, praecox forms are likely to be selected out. Praecox ammocoetes, derived from a fluviatilis population, will not have the advantage of longer river length to reduce flushing and the adults themselves bear the full migration mortality. As praecox adults can be expected to be smaller than fluviatilis adults due to the reduction of the adult feeding phase, their fecundity will be correspondingly lower. This lower fecundity is probably not sufficient to usually overcome the mortality.

In some situations however, praecox may survive in sufficient numbers to spawn. Short rivers will reduce migration time sufficiently to allow an effective adult feeding phase and if estuarine fish populations are large enough, it may be unnecessary for praecox to migrate out to sea. Such short rivers may not however, carry a large enough fluviatilis population in the first place and the praecox would not then arise in breeding numbers.

There is the possibility, mentioned above, that praecox forms could feed in freshwater. In most rivers insufficient prey would be available, but this possibility cannot be excluded.

With regard to the adaptive inferiority of praecox forms it must be emphasised that only individuals of praecox are needed. A population need not be maintained, and if it is not, selection will act only on single generations at a time and will not have the 'compound interest' effect that it exerts on continuous populations.

The number of planeri derived from the original fluviatilis population is small. It is possible that, say, 30 metamorphosed planeri are insufficient to produce a breeding population, especially if their origin is downstream of several tributary junctions and the adults disperse into different tributaries. If olfaction plays a part in the attraction of adults to spawning sites, however, the first few planeri up a stream will attract the rest and thus most of the 30 will spawn together. Such numbers are, by comparison with New Forest populations of planeri, probably large enough to produce persistent breeding populations.

The combination of circumstances that will allow the production of planeri by this Scheme (large fluviatilis populations; larval mortality reduced because of a series of years with only moderate rainfall; migration and adult mortality reduced because of unusually light predation and good food supplies) may not occur often in most rivers and never in some. However, the process postulated is a continuing one and in every year since the colonisation of a river by fluviatilis a certain number of praecox will arise. There have been 10,000 or more chances for the derivation of planeri since the Ice Age. It seems likely that many rivers during this period will have provided suitable conditions at least a few times.

Independent evolution of planeri types may occur after their origination. Intermediate praecox populations are unlikely to arise often and so gene flow between the fluviatilis and planeri, after the initial derivation of the latter, is likely to be only sporadic or non-existent. Populations of planeri in rivers from which fluviatilis have secondarily disappeared, or into which planeri have spread, will of course, evolve independently. Headwaters of rivers containing fluviatilis may be sufficiently isolated from fluviatilis-containing reaches to also allow the evolutionary independence of planeri once they have spread upstream.

Scheme II.

The second theory - the origin of planeri from a mixed homozygous and heterozygous fluviatilis population, would allow the initiation of planeri populations in all fluviatilis rivers every generation. This may account for the widespread distribution of planeri, small streams having been colonised by their dispersal.

However, planeri are only likely to be successfully derived from fluviatilis in upstream reaches. Planeri ammocoetes (aa-genotypes) arising in the lower reaches of the fluviatilis-colonised section will not have the advantages of reduced flushing or the larger number of available mudbanks that upstream lampreys will have. The very much lowered fecundity of the adult planeri is, under these circumstances, unlikely to compensate for the relatively high mortality. It is probable therefore, that only small, high-river-fluviatilis will be able to convey sufficient larval advantages to their aa planeri offspring.

Planeri may be expected, therefore, to arise only from upstream fluviatilis populations, but will be derived in relatively large numbers (¼ of the number of the upstream fluviatilis numbers) every generation.

The populations of fluviatilis that can successfully produce planeri populations may be those that are at the upstream limit of their ecological range, i.e. those whose high mortality from the long migration is only just countered by their rather low fecundity. Such fluviatilis populations are probably temporary, only persisting during periods when migration mortality is somewhat low. Planeri populations derived during these periods will become isolated on the disappearence of the fluviatilis population from high river reaches.

The possibility of indpendent evolution thus arises in these planeri populations, gene flow occurring from fluviatilis to planeri only when conditions favour the re-establishment of small, high-river fluviatilis.

At least partial genetic isolation of planeri populations can occur, therefore, with either Scheme. In the first, it is because praecox are relatively ill-adapted and will not usually reach sexual maturity in sufficient numbers to give a breeding population of praecox. In the second, it is because the high-river fluviatilis are the only ones that can provide sufficient larval advantage to their planeri offspring to ensure their survival to maturity and because these high-river fluviatilis are themselves usually ill-adapted to the prevailing conditions.

Populations of planeri, such as those in the New Forest, that are isolated from fluviatilis populations, will evolve independently and will further adapt to their environment. New Forest planeri are much smaller than planeri found in fluviatilis

containing rivers and this may represent an ecologicaly induced evolutionary trend in an isolated population. If the population were still in contact with the fluviatilis population from whence it was derived, it is possible that continuous recruitment of individuals would prevent such an evolutionary trend.

The second Scheme makes no allowance for the existance of a praecox form unless it is secondarily derivedby further mutation.

Both of these genetic modes of origin are open to investigation although the practical work involved would present difficulties. Two methods of approach are possible; the rearing to maturity of hybrids in captivity and the investigation of wild populations with the marking of ammocoetes for recapture as adults.

Planeri x fluviatilis hybrids will in the first Scheme give praecox adults. These should have a 5½ year ammocoete life, followed by migration and feeding in the adults. It is unfortunately almost impossible to rear adults through their marine phase, but in this case, if larval growth rates in the laboratory could be maintained at the same level as those of the wild populations, it ought not to be necessary. If all planeri x fluviatilis hybrids metamorphosed at 5½ year whilst planeri x planeri metamorphosed at 6½ years, and fluviatilis x fluviatilis at 4½, there would be good grounds for believing the intermediate to be the praecox form.

Ideally, F_2 crosses (praecox x praecox) should also be made. Offspring of these crosses should be in the ratio of 1 fluviatilis (4½ larval years): 2 praecox (5½y): 1 planeri (6½y). Again, the rearing to maturity is likely to be impossible in fluviatilis and praecox, but planeri could be kept to maturity and should breed truly.

In the second Scheme, Aa genotypes are likely to make up between ½ and ⅔ of the total fluviatilis numbers. Random hybridisation between fluviatilis should produce fluviatilis and planeri offspring in the ratio of about 11:1. In nature, the planeri offspring of most of the fluviatilis population would not survive, but the presumably reduced larval mortality under laboratory conditions should allow an approximation of these proportions to emerge. Planeri x fluviatilis (aa x AA or Aa) should give planeri and fluviatilis offspring in the proportions of about 3:1.

With the hybridisation experiments above, ammocoetes must be reared to metamorphosis and preferably through adult life. Equally important is that larval growth rates are as near to those found in wild populations as possible, especially where a praecox form with a 5½ year larval life is possibly involved.

Investigations of wild populations may help to support the first scheme if the existence of praecox can be shown. Unfortunately, praecox individuals will always be rather rare and will be indistinguishable, on most criteria, from fluviatilis.

Praecox ammocoetes will probably always be too few in number to be shown on length-frequency distribution analysis. Their 5½ year larval life is likely to be masked by planeri with its 6½ year ammocoete life.

Metamorphosing praecox will be larger than those of fluviatilis, but if silvery colouration is linked with the anadromous life history, they will be indistinguishable. It is, of course, possible that praecox macrophthalmia are frequently caught and are categorised as rather large fluviatilis macrophthalmia.

Adult praecox will be smaller than average fluviatilis, but again are likely to be indistinguishable from small fluviatilis. Small animals presently identified as fluviatilis may in fact be praecox, just as large fluviatilis macrophthalmia may be praecox. Unless there is a discontinuity in the range of sizes, which seems improbable, neither macrophthalmia nor adults of praecox could be distinguished from those of fluviatilis.

There is, ex-hypothesis, no phenotypic difference between AA and Aa fluviatilis in the second Scheme. Were there to be so, selection would alter the frequency of the gene a, and although this would not necessarily invalidate the Scheme, it would make the theoretical implications unnecessarily complicated.

The marking of ammocoetes for recapture.

There is at present no suitable method of marking ammocoetes for recapture as adults. It is possible that the implantation of radio-isotopes may be fruitful, but such an isotope would have to be metabolically inert, be retained in the organism for a long period of time, have a suitable half-life and its emissions would have to be as soft as possible, although compatible with detection. The major disadvantage of radioactive marking is that mutation rates may be affected and this would prejudice the value of the interpretation of any such experiments.

If a suitable method of marking can be developed however, it would prove very useful in the clarification of the genetic mechanisms proposed here.

In the first Scheme, labelling of large 'fluviatilis' macrophthalmia might show a positive correlation between 'largeness'

of macrophthalmia and 'smallness' of adult size, i.e. the praecox form.

With the praecox intermediary mechanism proposed in the first Scheme, a combination of hybridisation and marking with release and recapture would probably prove the most useful technique. Thus, planeri x fluviatilis ammocoetes should be recaptured as small 'fluviatilis' (= praecox), whilst praecox x praecox crosses (obtained from planeri x fluviatilis crosses) should give ammocoetes that mature as fluviatilis, praecox, or planeri. The advantages of release of hybrids is that more or less natural growth and mortality conditions are assured, as is the provision of marine conditions for anadromous adults; always provided that the marking method has no deleterious effect.

In the second Scheme, marked planeri x fluviatilis hybrids should be recovered as fluviatilis adults only; the marking of fluviatilis x fluviatilis ammocoetes also necessitates artificial fertilisation, as fluviatilis ammocoetes are indistinguishable from those of planeri. Fluviatilis x fluviatilis crosses should provide adults that are both fluviatilis and planeri, but the necessity of releasing ammocoetes far upstream must be borne in mind.

The Schemes outlined above are not open to investigation at every stage, but it is hoped that where clarification is possible it will be undertaken.

These Schemes depend upon the timing of metamorphosis being controlled by a simple genetic change. The period of time available for such a change necessitates that the change was quick and therefore simple.

The development of brook lampreys seems to be a common trend in lamprey evolution throughout the world. The major areas containing lampreys have been affected by the Pleistocene Glaciation (including Australia, Browne, 1957). The circumstances that have led to the derivation of planeri will have obtained in these areas also.

It may be supposed that the original pre-Pleistocene parasitic populations will have given rise to non-parasitic forms for the reasons outlined above. During the Pleistocene Glaciation some of the non-parasitic population will have been destroyed whilst those in the regions beyond the limits of periglacial conditions will have survived. These populations are probably now represented by the relict and 'degenerate' non-parasitic forms found in southern North America, Mexico, southern Europe and south eastern Asia.

After the retreat of glacial conditions the parental parasitic populations will spread polewards and the present day non-parasitic paired species will have been derived in situ since this time.

At this stage it is impossible to decide whether planeri and fluviatilis are specifically different. The mechanisms outlined above will allow eventual speciation, but it is possible that the populations investigated have not yet reached this stage.

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